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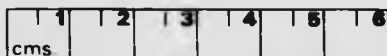
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Cloning and Characterisation of Developmental  
Genes in Myxococcus xanthus using a Plasmid  
based Promoter Probe

David E. Hartree, B.A. Oxford, M.Sc. Sussex

A thesis submitted for the degree of Doctor  
of Philosophy at the University of Warwick

Microbiology Group  
Department of Biological Sciences  
University of Warwick  
Coventry CV4 7AL  
U.K.

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# Contents

SECTION	TITLE	PAGE NO.
PREFACE		
	Contents	i
	List of figures	vii
	List of tables	xi
	Acknowledgements	xiii
	Declaration	xiv
	Summary	xv
	Abbreviations	xvi
	Dedication	xviii
CHAPTER 1		
	Introduction	
1-1	The Myxobacteria	2
1-2	Developmental biology of <u>Myxococcus xanthus</u>	4
1-2-1	Description of <u>Myxococcus xanthus</u>	4
1-2-2	Developmental cycle of <u>M. xanthus</u>	6
1-2-3	Fruiting bodies	9
1-2-4	Spore formation	9
1-2-5	Glycerol spores	11
1-3	Genetic systems in <u>M. xanthus</u>	12
1-3-1	Introduction	12
1-3-2	Myxophages	12
1-3-3	Phage P1 and derivatives	13
1-3-4	Homology based integration	14
1-3-5	Design of homology based promoter probes	17
1-3-6	Advantages of homology based promoter probes	24
1-3-6-1	The existing gene is not inactivated	24
1-3-6-2	Ease of cloning	24

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96



1-3-6-3	Mutagenesis of the cloned genes	27
1-4	Genetics of <u>Myxococcus</u> development	28
1-4-1	Introduction	28
1-4-2	Motility mutants	28
1-4-3	Sporulation mutants	30
1-4-4	Aggregation mutants	31
1-4-5	Glycerol resistant mutants	33
1-4-6	Reverse genetics	33
1-4-7	Gene fusions	34
CHAPTER 2		
Materials and methods		
2-1	Strains used	39
2-1-1	<u>E. coli</u> K12	39
2-1-2	<u>E. coli</u> phages	39
2-1-3	Existing <u>M. xanthus</u> strains	40
2-1-4	<u>M. xanthus</u> strains prepared during the course of this work	41
2-2	Plasmids	41
2-2-1	Existing plasmids	41
2-2-2	Plasmids prepared during the course of this work	42
2-2-3	Genes characterised in the course of this work; their nomenclature	43
2-2-4	<u>In5</u> inserts into <u>M. xanthus</u>	43
2-3	Chemicals, media and antibiotics	44
2-3-1	Chemicals	44
2-3-2	Media	44
2-3-3	Buffers	45
2-3-4	Antibiotics	47
2-4	Growth and maintenance of cultures	48
2-5	Preparation of phage lysate	48

2-5-1	P1 lysates	48
2-5-2	Lambda lysates	49
2-6	P1 mediated transduction of <u>M. xanthus</u>	50
2-6-1	Packaging of plasmid into P1	50
2-6-2	P1 transduction of <u>M. xanthus</u>	51
2-7	Tn5 mutagenesis of plasmids in <u>E. coli</u>	51
2-8	<u>gamma-delta</u> mutagenesis in <u>E. coli</u>	52
2-9	Testing for sporulation in <u>M. xanthus</u>	53
2-10	Induction of sporulation by starvation	54
2-11	Chemical induction of sporulation	54
2-12	Sonication of <u>M. xanthus</u> samples	55
2-13	Protein assay	56
2-14	$\beta$ -galactosidase detection and assays	56
2-15	DNA extraction from <u>M. xanthus</u>	58
2-15-1	Preparation of chromosomal DNA	58
2-15-2	Preparation of closed circular DNA from <u>M. xanthus</u>	59
2-16	Isolation of plasmid from <u>E. coli</u>	60
2-16-1	Rapid, small-scale isolation of plasmid DNA	60
2-16-2	Large-scale preparation of plasmid DNA	61
2-17	Transformation of <u>E. coli</u>	61
2-18	Restriction endonuclease digestion	62
2-19	Alkaline phosphatase	62
2-20	Use of Exonuclease III to create deletion mutants of plasmids	63
2-21	Ligations	64
2-22	Labelling of DNA by nick-translation	64
2-23	Phenol extraction	65
2-24	Ethanol precipitation	65
2-25	Agarose gel electrophoresis	68
2-26	Elution of DNA from agarose gels	68

2-27	Southern transfer	68
2-28	Colony transfer	69
2-29	Hybridisation conditions	69

## CHAPTER 3

Preparation and identification of developmentally regulated gene fusions

3-1	Introduction	72
3-2	Cloning of <u>M. xanthus</u> chromosomal DNA	72
3-3	Transductants of <u>M. xanthus</u> show differential expression of $\beta$ -galactosidase	81
3-4	Attempts were made to isolate additional gene fusions of interest	99
3-5	Cloning of gene fusions	100
3-5-1	Circular DNA, spontaneously formed in <u>M. xanthus</u> , can be cloned in <u>E. coli</u>	100
3-5-2	The cloned gene fusions could be retransduced intact to <u>M. xanthus</u>	101
3-6	The strain UWMS contains a mutant allele affecting aggregation	105
3-7	Concluding remarks	108

## CHAPTER 4

The effect of mutant genetic background in M. xanthus upon the expression of gene fusions

4-1	Introduction	112
4-2	Transduction of gene fusions into mutant strains	113
4-3	Assay conditions	113
4-4	Expression of <u>isg-2&gt;lacZ</u> in different genetic backgrounds	114
4-5	Expression of <u>isg-1&gt;lacZ</u> in different genetic backgrounds	127
4-6	Expression of pUWM2 in different genetic backgrounds	136

4-7	Concluding remarks	137
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## CHAPTER 5

Restriction mapping of the regions surrounding gene fusions PUWM4, PUWM5, A45 and D32, and attempts to clone the region extending past the gene fusion

5-1	Introduction	146
5-2	Cloning the chromosomal regions extending past the gene fusions	146
5-3	Mapping of the cloned genes in PUWM4, PUWM4', PUWM5, PUWM5', pD32 and pD32'	147
5-4	Southern analysis of the <u>isg-2</u> region	167
5-5	Concluding remarks	176

## CHAPTER 6

Site directed mutagenesis of the isgA1 and isgB2 genes and their surrounding regions

6-1	Introduction	179
6-2	The principle of <u>Tn5</u> mutagenesis	180
6-3	Preparation of targets for mutagenesis	186
6-4	Mutagenesis of plasmids PUWM4'tc and PUWM5'D23 in <u>E. coli</u>	188
6-5	Mapping the positions of <u>Tn5</u> inserts	193
6-6	Preparation of <u>Tn5</u> inserts in <u>M. xanthus</u> through gene replacement by plasmid borne <u>Tn5</u> inserts	194
6-7	Phenotypic analysis of mutants	205
6-8	Concluding remarks	206

## CHAPTER 7

Analysis of the functional regions of gene fusions by means of site-directed mutagenesis

7-1	Introduction	212
7-2	Transduction of mutated gene fusions into <u>M. xanthus</u> ; the principles involved	213
7-3	Use of transposon <u>gamma-delta</u>	218
7-3-1	<u>gamma-delta</u> mutagenesis of <u>isgA1&gt;lacZ</u> and <u>isgB2&gt;lacZ</u>	218

7-3-2	Transduction of <u>M. xanthus</u> using PUWM40 containing <u>gamma-delta</u> insertions	223
7-4	Mutagenesis of <u>isgB2&gt;lacZ</u> using Tn5	234
7-4-1	Preparation of Tn5 inserts within PUWM5	234
7-4-2	Transduction of <u>M. xanthus</u> using PUWM5::Tn5(-1300)	237
7-5	Mutagenesis of the gene fusion <u>isgB2&gt;lacZ</u>	243
7-5-1	Insertion of the omega fragment upstream of the gene fusion <u>isgB2&gt;lacZ</u>	243
7-5-2	Transduction of <u>M. xanthus</u> using PUWM50::Ω	243
7-6	Concluding remarks	254

#### CHAPTER 8

Overall discussion: The relevance of results obtained to the understanding of microbial development; possible future experiments.

8-1	The promoter-probe library of <u>M. xanthus</u> chromosomal DNA	258
8-2	The screening procedures employed	259
8-3	Expression of gene fusions in different backgrounds	261
8-4	Use of site directed mutagenesis in determining the functional regions of genes and the position of gene promoters	265
	References	268

## List of figures

FIGURE	TITLE	PAGE
1-1	Site directed mutagenesis of <u>M. xanthus</u> by gene replacement	16
1-2	Construction of a gene library in a plasmid based promoter probe	19
1-3	Packaging of plasmid into P1 phage for transduction of <u>M. xanthus</u>	21
1-4	The two possible outcomes of the integration of a homology based promoter probe	23
1-5	Cloning of a gene for which a gene fusion has been obtained	26
1-6	Epistasis map of developmentally regulated gene fusions	37
2-1	Spun column technique used to purify DNA labelled by nick translation	67
3-1	Plasmids pDAH122 and pDAH283	74
3-2	Sequence of the <u>trp-lac</u> region	76
3-3	Partial digestion of <u>M. xanthus</u> DNA for cloning in a promoter probe plasmid	80
3-4-1	Expression of gene fusion in strain UWM1 during starvation induced sporulation	88
3-4-2	Expression of gene fusion in strain UWM1 during glycerol induced sporulation	88
3-5-1	Expression of gene fusion in strain UWM2 during starvation induced sporulation	90
3-5-2	Expression of gene fusion in strain UWM2 during glycerol induced sporulation	90
3-6-1	Expression of gene fusion in strain UWM3 during starvation induced sporulation	92
3-6-2	Expression of gene fusion in strain UWM3 during glycerol induced sporulation	92
3-7-1	Expression of gene fusion in strain UWM4 during starvation induced sporulation	94

3-7-2	Expression of gene fusion in strain UWM4 during glycerol induced sporulation	94
3-8-1	Expression of gene fusion in strain UWM5 during starvation induced sporulation	96
3-8-2	Expression of gene fusion in strain UWM5 during glycerol induced sporulation	96
3-8-3	Expression of gene fusion in strain UWM5 and DK101[pUWM5] during glycerol induced sporulation	98
3-9	Restriction analysis of plasmids formed by spontaneous recombination within the <u>M. xanthus</u> chromosome	103
3-10	A sporulation/aggregation mutation in UWM5	107
4-1-1	Expression of <u>isg-2&gt;lacZ</u> during development of DK1622[pUWM5]	116
4-1-2	Expression of <u>isg-2&gt;lacZ</u> during development of DK306(Mgl)[pUWM5]	118
4-1-3	Expression of <u>isg-2&gt;lacZ</u> during development of DK5057(Asg)[pUWM5]	120
4-1-4	Expression of <u>isg-2&gt;lacZ</u> during development of DK3621(Dsg)[pUWM5]	120
4-1-5	Expression of <u>isg-2&gt;lacZ</u> during development of LS202(Csg)[pUWM5::Tn5(1300)]	122
4-1-6	Expression of <u>isg-2&gt;lacZ</u> during development of DK101[pUWM5]	122
4-1-7	Expression of <u>isg-2&gt;lacZ</u> during development of UWM5	124
4-1-8	Bar chart summarising <u>isg-2&gt;lacZ</u> expression in different backgrounds	127
4-1-9	Bar chart showing range of <u>isg-2&gt;lacZ</u> expression in different backgrounds	127
4-2-1	Expression of <u>isg-1&gt;lacZ</u> during development of DK1622[pUWM4]	131
4-2-2	Expression of <u>isg-1&gt;lacZ</u> during development of DK306(Mgl)[pUWM4]	131
4-2-3	Expression of <u>isg-1&gt;lacZ</u> during development of DK5057(Asg)[pUWM4]	133

4-2-4	Expression of <u>isg-1</u> > <u>lacZ</u> during development of DK3621(Dsg)[PUWM4]	133
4-2-5	Bar chart summarising <u>isg-1</u> > <u>lacZ</u> expression in different backgrounds	135
4-3	Expression in <u>M.xanthus</u> strains carrying PUWM2	139
5-1-1	Map of plasmid PUWM4	149
5-1-2	Map of plasmid PUWM4'	151
5-2-1	Map of plasmid PUWM5	153
5-2-2	Map of plasmid PUWM5'	155
5-3-1	Map of plasmid pD32	157
5-3-2	Map of plasmid pD32'	159
5-4-1	Southern analysis of <u>isg-2</u> region	171
5-4-2	Map of <u>isg-2</u> region	173
5-5	Map of plasmid pDAH122	175
6-1	Possible outcomes of the transduction of <u>M.xanthus</u> sequences containing <u>Tn5</u> into <u>M.xanthus</u>	182
6-2	Method for obtaining deletions in the kanamycin resistance gene	185
6-3	Method for replacing plasmid borne kanamycin resistance with tetracycline resistance	190
6-4	<u>Tn5</u> inserts in PUWM4'tc	196
6-5	<u>Tn5</u> inserts in PUWM5'D23	198
6-6	Two ways in which <u>isg82</u> could escape inactivation by <u>Tn5</u>	208
7-1	Possible outcomes of the transduction of a gene fusion containing a transposon	216
7-2	Size reduction of plasmids containing gene fusions	221
7-3	Positions of <u>gamma-delta</u> inserts in PUWM4D	227
7-4	Interpretation of Southern analysis upon strains transduced by <u>gamma-delta</u> derivatives of PUWM4	230



7-5	Construction of pUWM5 derivatives containing Tn5	236
7-6	Interpretation of Southern analysis upon strains transduced by Tn5 derivatives of pUWM5	242
7-7	Construction of pUWM5D:: $\Omega$	245
7-8	Southern analysis of strains transduced with pUWM5D:: $\Omega$	251
7-9	Interpretation of Southern analysis of strains transduced with pUWM5D:: $\Omega$	253

## List of tables

TABLE	TITLE	PAGE
1-1	A classification of the myxobacteria	3
1-2	Sporulation mutants of <u>M. xanthus</u> ; properties of the four complementation groups	32
3-1	A chromosomal library in a plasmid based promoter probe; Numbers of transformants obtained	78
3-2	Effect of nutrient conditions upon the expression of different gene fusions	78
3-3	Promoter probe libraries; Their transduction to <u>M. xanthus</u>	83
3-4	The sporulation dependent expression of a variety of promoter probe fusions	85
4-1	Statistical analysis of developmentally regulated expression of pUWM2	141
5-1-1	Restriction analysis of pUWM4	161
5-1-2	Restriction analysis of pUWM4'	162
5-2-1	Restriction analysis of pUWM5	163
5-2-2	Restriction analysis of pUWM5'	164
5-3-1	Restriction analysis of pD32	165
5-3-2	Restriction analysis of pD32'	166
5-4	Southern analysis of <u>isg-2</u> region	169
6-1	Mutagenesis of pUWM5'D23 in <u>E.coli</u> using <u>Tn5</u>	192
6-2	Mutagenesis of pUWM4'tc in <u>E.coli</u> using <u>Tn5</u>	192
6-3	Mutagenesis of DK1622 using gene replacement by a pUWM4 mutant	201
6-4	Mutagenesis of DK1622 using gene replacement by a pUWM5 mutant	201
6-5	Mutagenesis of DK306 using gene replacement by a pUWM5 mutant	201
6-6	Southern analysis of strains carrying possible <u>Tn5</u> inserts ( <u>isgB2</u> region)	202

6-7	Southern analysis of strains carrying possible <u>Tn5</u> inserts ( <u>isgA1</u> region)	204
7-1	Plasmids used in transduction experiments in this chapter	214
7-2	Transductant <u>M. xanthus</u> strains with <u>gamma-delta</u> ; the effect upon the gene fusion <u>isgA1</u> > <u>lacZ</u>	225
7-3	Southern analysis of strains containing <u>gamma-delta</u>	231
7-3-1	Probe PUWM4	231
7-3-2	probe <u>gamma-delta</u>	232
7-4	$\beta$ -galactosidase expression of strains carrying PUWM5:: <u>Tn5</u> (-1300)	238
7-5	Southern analysis of strains containing PUWM5:: <u>Tn5</u> (-1300)	239
7-6	$\beta$ -galactosidase expression of strains carrying PUWM5D:: $\Omega$	247
7-7	Southern analysis of strains obtained by transduction of DK306 using PUWM5D:: $\Omega$	248

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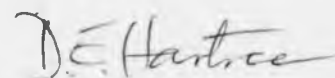
I acknowledge the financial support of a SERC Instant Award for the three years of this project.

## Declaration

All the results in this thesis are from my own work except where stated otherwise.

Sources of information, published and unpublished, have been acknowledged in the text and bibliography.

No part of this work has been previously submitted as a thesis towards a higher degree.

A handwritten signature in dark ink, appearing to read 'D.E. Hartree', with a stylized, cursive script.

D. E. Hartree

## Summary

An integrative promoter-probe plasmid was used as a cloning vector for randomly cut DNA from *Myxococcus xanthus*. The resulting chromosomal library of gene fusions to the lacZ gene was returned to *M. xanthus* using P1 mediated transduction. Transductants were screened for  $\beta$ -galactosidase expression under both vegetative and sporulation conditions. Several strains were found which showed increases in expression during sporulation. This indicated that the lacZ gene had become fused to a sporulation gene.

More detailed studies were carried out upon two of the gene fusions. Fusion isgH1>lacZ was transduced into other genetic backgrounds including backgrounds with known mutations which block sporulation. The gene fusion showed increased expression under starvation conditions even in mutants unable to sporulate. The isgH1 gene, therefore, appears to be activated early during development. A second gene fusion isgB2>lacZ was found to be expressed strongly in circumstances where spores were forced to form outside of fruiting bodies. It would, therefore, appear to be a conditional sporulation gene. There is virtually no increase in activity when sporulation occurs in a wild type background.

Experiments were carried out using transposon mutagenesis of the isgH1 and isgB2 genes. No mutant strains could be obtained with insertions in the region of the isgH1 gene. This suggests that the region is essential to the growth of the cell. Insertions were readily obtained in the isgB2 region. However, no mutant phenotype was observed. Transposon mutagenesis upon the isgB2>lacZ fusion was employed to locate the isgB2 promoter. The location of the promoter was defined to within a region of 700 base pairs.

## Abbreviations

NB. Abbreviations for media, antibiotics and buffers are described elsewhere (in Materials and Methods).

n	(followed by subscript of wavelength in nanometres) Absorbance
bp	Base pairs
C	Cytosine
°C	Degrees Celsius
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetracetic acid
G	Guanine
GTP	Guanosine triphosphate
Kb	Kilobase pairs
MBHA	Myxobacterial haemagglutinin
min	Minutes
M.O.I	Multiplicity of infection
4-MUG	4-Methylumbelliferyl galactoside
ONP	Ortho-nitrophenol
ONPG	Ortho-nitrophenyl galactoside
PEA	Phenylethyl alcohol
pfu	Plaque forming unit
ppGpp	Guanosine tetraphosphate
r	(superscript with antibiotic abbreviation) Resistant
RNA	Ribonucleic acid

rpm	Revolutions per minute (centrifugation)
rRNA	Ribosomal RNA
s	(superscript with antibiotic abbreviation) Sensitive
SDS	Sodium dodecylsulphate
Tris	Tris(hydroxy)aminomethane
Tris.Cl	Tris(hydroxy)aminomethane adjusted in pH by adding HCl
TTP	Thymidine triphosphate
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ - D-galactoside



Dedication

This thesis is dedicated to my parents whose encouragement and support I acknowledge, not only during this project but for the whole of my academic career.

## CHAPTER 1

### Introduction

### 1-1 The Myxobacteria

Myxobacteria are Gram-negative bacteria which possess gliding motility. All are obligate aerobes. In nature they exist either as swarms of vegetative cells or as fruiting bodies filled with spores. The degree of cellular cooperation required to form the fruiting bodies is remarkable for a prokaryote and there is considerable interest in myxobacteria as models for development and differentiation (Rosenberg E., 1984). Also remarkable, is the cooperative swarming of vegetative cells, which increases their ability to digest complex organic substrates (Rosenberg E. et al. 1977). With the exception of one genus Sorangium, which can digest cellulose, the myxobacteria digest proteins and cannot use carbohydrate as a main carbon source (Reichenbach H. and Uworkin M. 1981). The Myxobacteria appear to be a genuine taxonomic group (Ludwig W. et al. 1977) on the basis of rRNA homology. Furthermore, their raised multicellular fruiting bodies are unique among prokaryotes and they have similar DNA base ratios (GC around 70%). A classification of myxobacteria is shown on Table 1-1.

TABLE 1-1

Taxonomic survey of the Myxobacteria  
From Reichenbach H. and Dworkin M., (1981)

Order: **Myxobacteriales**Suborder: **Cystobacterineae**

Families and  
genera:

## Myxococcaceae

MyxococcusCorallococcusAngiococcus

## Archangiaceae

Archangium

## Cystobacteraceae

CystobacterMelittangiumStigmatellaSuborder: **Sorangineae**

Families and  
genera:

## Sorangiaceae

SorangiumPolyangiumHaploangiumChondromycesNannocystis

## 1-2 Developmental biology of Myxococcus xanthus

### 1-2-1 Description of Myxococcus xanthus

Myxococcus xanthus in the vegetative state, exists as rod shaped cells, up to 7µm long and 0.7µm across. The fruiting bodies, which are fully formed 48 hours after the onset of starvation, are spherical and around 100µm across. These contain spherical spores around 2µm in diameter. The spores of M. xanthus are not as resistant to adverse conditions as those of some bacteria. Nevertheless, they can withstand temperatures up to 60°C, UV irradiation and desiccation (Sudo S.Z. and Dworkin M. 1969.)

Myxococcus xanthus secretes a variety of extracellular enzymes such as proteases, amidases and glucosamidases (Rosenberg E. and Varon M. 1984). These enable them to break down the cell walls and proteins of the bacteria upon which they feed. The breaking of cell walls is enhanced by contact with the M. xanthus cell surface, or with its secreted slime layer which entraps secreted enzymes. In addition, M. xanthus secretes an antibiotic TA. This is active against a wide range of bacteria, and lyses cells by interfering with peptidoglycan synthesis (Rosenberg E. and Varon M. 1984).

The cooperative swarming behaviour of M. xanthus is important for efficient proteolysis. For example, when grown on a liquid medium containing the protein casein, cell density must be at least 10<sup>8</sup> cells/ml for growth to occur. On hydrolysed casein (casitone), growth is independent of cell

density (Rosenberg E. et al. 1977). Amino acids would appear to be the main carbon source for M. xanthus. Carbohydrates are not apparently usable as a sole carbon source but can be co-metabolised with proteins (Bretcher A.P. and Kaiser D. 1978).

Gliding is the ability to move across surfaces without the need for flagella and is a property shared with all other myxobacteria and several unrelated groups such as some cyanobacteria and Cytophaga spp. Gliding bacteria invariably secrete extracellular slime, a complex of proteins, polysaccharides and lipids. It seems unlikely that directed release of slime is the mechanism of gliding (Burchard R.P. 1984). It is possible that gliding occurs by directed release of surfactant giving rise to changes in surface tension (Dworkin M. et al. 1983). However, observations of motility and fruiting body formation underwater (Kuner J.M. and Kaiser D. 1982) would seem to disprove this theory. Other theories involve hypothetical contractile organelles in the cells. These could cause localised shearing of the slime and loss of adhesiveness. A wave of such contractions could propel the cell forwards. Contractile mechanisms have not been shown to exist in M. xanthus. However, they have been recorded in the unrelated gliding species, Flexibacter (Burchard R.P. 1982). Another unrelated gliding species (Cytophaga sp.) has also been investigated. The outer membrane was shown to anchor polystyrene latex spheres which were then propelled in either direction along the length of the cell (Lapidus I.R. and Berg H.C. 1982). Actin-like proteins have been found in some bacteria although not myxobacteria, and is a further indication of the existence of contractile mechanisms in

prokaryotes (Gilbert M. and Fulton R.B. 1982).

The cell envelope of M. xanthus is typical of the Gram negative bacteria in having inner and outer membranes separated by a peptidoglycan layer. Trypsin digestion releases patches of peptidoglycan (White D. et al. 1968). This contrasts with the continuous sacculus of peptidoglycan released from trypsin digested Escherichia coli. Such an arrangement may allow the change in shape from rod shaped cell to spherical spore. It may also be of relevance to the gliding motility already mentioned.

The genome size of M. xanthus has been determined using 2D DNA electrophoresis with in situ digestion (Yee T, Inouye M. 1984). It lies in the range of  $5690 \pm 510$  Kb. The GC content is high, about 70%. Two minute extrachromosomal elements are found, each at high copy number. They have extensive secondary structure with single stranded regions and consist of both RNA and DNA, and are homologous to genomic sequences (Dhundale R. et al. 1987ab). No plasmids have been found in M. xanthus.

#### 1-2-2 Developmental cycle of M. xanthus

Vegetative cells of M. xanthus undergo development if three conditions prevail: i) They are starved; ii) They are on a surface where gliding is possible; and iii) The cells are at a sufficiently high density. Starvation is the metabolic prerequisite for development. The cessation of growth for any other reason, such as the cells entering stationary phase, does not initiate development. Starvation for any required amino acid (leucine, isoleucine, valine or phenylalanine) can

induce development. Auxotrophic mutants requiring an extra amino acid will undergo development if that amino acid is lacking. Starvation of a limiting energy source even when this is not an amino acid, for example pyruvate, induces development, as does the depletion of inorganic phosphate from the environment (Manoil C. and Kaiser D. 1980).

The mechanism which triggers development is not known. Like E. coli, there is a stringent response after starvation. There is a sharp rise in levels of guan tetraphosphate (ppGpp) and guan pentaphosphate (pppGpp). In E. coli, ppGpp alters the pattern of gene expression both in vivo and in vitro. In the case of Bacillus, the trigger for sporulation would appear not to be the increase in guanine polyphosphates but the accompanying decrease in GTP levels (Lopez J.M. et al. 1981). Myxococcus exhibits increased ppGpp levels during starvation, yet GTP levels are not significantly reduced. However, the significance of this for the initiation of development is not clear. Where cells are grown on a minimal medium the ppGpp pool remains high, yet development does not take place (Manoil C. and Kaiser D. 1980). For coordinated development of fruiting bodies, not only must individual cells respond to changing conditions, but cells must be able to communicate with one another. Diffusible substances from fruiting bodies have been shown to cause fruiting in vegetative cells. Such substances can diffuse through agar (Lev M. 1954) and through cellophane (McVittie A. and Zahler S.A. 1962) and they can be species specific. However, such results are not necessarily evidence for intercellular signalling. The diffusible molecules could be metabolic intermediates whose presence allows the normal inhibition of



development to be bypassed.

Cells must be above a critical density for sporulation to occur. They must, therefore, have some means of determining their own density. There is some evidence that adenine is involved (Shimkets L.J. and Dworkin M. 1981). If cells at too low a density to aggregate are supplied with adenine or adenine containing compounds, aggregation and fruiting can occur. Furthermore, hadacidin, which inhibits AMP synthesis and Norit which binds purines, both inhibit aggregation. It is possible that these are nonspecific effects brought about by the change in the energy charge of the cell. However, other nucleotides do not have the same effect. Furthermore, in another myxobacterium, Stigmatella aurantiaca, it is guanine which can initiate aggregation while adenine has no effect (Stephens K. and White D. 1980).

Fruiting bodies of any one species are of characteristic shape. Cells must be directed towards a centre of aggregation in a controlled manner. Little is known of the mechanisms for this (Burchard R.P. 1984). Chemotaxis has been suggested. However, observations of cells moving along an artificial chemical gradient or moving around obstacles, can be explained as elastictaxis. This is a process where cells follow lines of stress on an agar surface. Aggregation and sporulation can occur under water (Kuner J.M. and Kaiser D. 1982). This makes the existence of a gradient in the medium seem unlikely, although a signal relay between cells in contact would be possible.

Another possibility is that the form and distribution of fruiting bodies is a product of the motility properties of the cells and the physical properties of the cells and their

slime. Certain mutants, with altered motility, produce aberrant fruiting bodies (Blackhart B.D. and Zusman D.R. 1985). The slime coating the cells could also have an important role. A complex polysaccharide can change between fluid and gel depending upon ionic concentrations and in response to shearing forces (Rees D.A. 1972).

#### 1-2-3 Fruiting bodies

Widespread cell lysis has been reported to occur within fruiting bodies (Wireman J. and Dworkin M. 1977). It has been suggested that the lysed cells provide spore components to the surviving cells and that lysis is an essential part of the developmental cycle. The situation would be analogous to the endospore and mother cell of Bacillus. Recent examination of the evidence suggests that large scale cell lysis does not occur during development (O'Connor K. and Zusman D.R. 1988). Thorough examination of fruiting bodies has revealed that large scale cell loss is not a necessary part of development and that previous evidence of lysis was an artefact of harvesting conditions.

#### 1-2-4 Spore formation

The formation of spores in fruiting bodies requires the massive synthesis of spore components. There is a considerable change in the pattern of protein synthesis. A quarter of the soluble proteins, resolvable by polyacrylamide gel

electrophoresis, show changes in abundance (Inouye M. et al. 1979). New RNA species are synthesised some of which have unusually long half-lives (Nelson D.R. and Zusman D.R. 1983).

One spore protein is produced in extremely large quantities. It is known as protein 5 (Inouye M. et al. 1979). It first appears 3-6 hours after the start of development, and the rate of synthesis increases until 24 hours when it comprises 15% of the total soluble protein. By 48 hours, its production has decreased to zero. Its molecular weight is 19000 daltons. Although initially a cytoplasmic protein, it later forms the outer spore coat. The protein, solubilised by treatment with EDTA, can reassemble spontaneously onto the spore surface in the presence of calcium ions. The gene for protein 5 has been cloned and sequenced. It lacks any apparent sequences which could code for a leader peptide (Inouye et al. 1983). The protein could be released during the developmental lysis of other cells (if this actually occurs) or there may be specialised translocation mechanisms. Another protein of interest is a development specific lectin, myxobacterial haemagglutinin (MBHA) (Cumsky M.G. and Zusman D.R. 1979). It is present only in cells undergoing fruiting body formation. It is localised on the cell surface and in the periplasmic space (Nelson D.R. et al. 1981).

The commitment of cells to sporulate does not occur until well after the initiation of development (Wireman J. 1979). Cells from fruiting bodies induced by starvation normally form spores after 40-50 hours. They can resume growth when put into nutrient (Casitone) medium at any time up to 36 hours. After this stage, they form spores even after transfer to rich medium.

#### 1-2-5 Glycerol spores

Sporulation can be induced artificially by adding certain low molecular weight organic compounds, such as glycerol or dimethylsulphoxide (DMSO), to log phase cultures. This would appear to activate the later part of the sporulation pathway. It does not require starvation or the presence of a solid surface. Certain spore components are absent, such as protein S. Glycerol induced spores have a thin, single layered coat and are less resistant to heat and sonication than are normal spores (Sudo S.Z. and Dworkin M. 1969). Another difference is their relatively high rate of respiration (up to 20% that of vegetative cells). Glycerol spores can form in about 2 hours and their formation is independent of the cell cycle. Much research on sporulation has been carried out using this system (Zusman D. 1984), as it is rapid and synchronous. The mechanism of glycerol induction is uncertain. Little or no inducer is taken up into the cell, so it is likely to act directly upon the cell envelope (Sadler W. and Dworkin M. 1966). Interestingly, phenethyl alcohol (PEA) appears to induce sporulation differently. There are some differences in the proteins produced with PEA than with glycerol or DMSO (Komano T. et al. 1980). Moreover, there are mutants which cannot be induced with glycerol or DMSO but can be with PEA (Burchard R.P. and Parish J.H. 1975).

### 1-3 Genetic systems in M. xanthus

#### 1-3-1 Introduction

Genetic studies in M. xanthus were hampered for many years because of the way most isolates grow as a cohesive swarm rather than as dispersed cells. Furthermore, no plasmids are known to exist in the species nor is there any conjugation. The first breakthrough was the establishment of dispersed growing strains (Reichenbach H. and Dworkin M. 1981). This made possible both the isolation of mutant strains and the selection of transductants. Three phages are known which can transduce M. xanthus. Another transducing phage is the coliphage P1. This can adsorb to the cell and inject its DNA but infection is unable to proceed further. This is used as a suicide vector to force DNA into the cell. Another approach has been to mate strains of M. xanthus with a donor E. coli strain. Conjugative plasmids of the incP group can be transferred to M. xanthus, where they can integrate into the chromosome by site-specific recombination (Jaoua S. et al. 1987).

#### 1-3-2 Myxophages

Phage Mx8 (Martin S. et al.) is the most commonly used transducing phage in M. xanthus. It is a temperate phage with a genome of 95kb and has a unique attachment site in the host

chromosome. It can package host DNA instead of its own and is, therefore, capable of generalised transduction. Any selectable phenotype can be transduced between strains using this phage.

#### 1-3-3 Phage P1 and derivatives

Coliphage P1 binds to a lipopolysaccharide receptor (Lindberg A. 1973) and this enables it to infect M. xanthus. The infection is not productive; there is no lysogeny nor are any progeny phage produced. If the phage genome carries transposon Tn5, this can transpose onto any point on the M. xanthus chromosome and is selectable by conferring kanamycin resistance. Further transposition is very infrequent. In this way, a selectable marker can be placed next to any gene of interest. This has been used to characterise mutant alleles and assign them to particular loci (Kuner J.M. and Kaiser D. 1981).

Derivatives of Tn5 are also available. The sequences required for transposition are confined to the IS50R region. The IS50L region is defective and nearly all of it can be deleted without affecting transposition. Such a property allows considerable scope for inserting novel sequences into M. xanthus. One derivative of Tn5 is TnV, which has a plasmid replication origin in addition to a kanamycin resistance marker (Furuichi T. et al. 1985a). This has been used to create developmental mutants of M. xanthus. The mutated region could then be cloned as a plasmid in E. coli in one step.

Another approach has been to insert genes coding for an easily assayable enzyme but lacking the gene promoter region.

his intervening Tn5 transcription terminators have been removed, it is possible to detect activity from an adjacent M. xanthus promoter. A promoter-probe of this kind has been constructed using the lac Z gene which codes for  $\beta$ -galactosidase (Kroos L. and Kaiser D. 1984).

#### 1-3-4 Homology based integration

An alternative to random mutagenesis of the entire chromosome, using Tn5, is the use of integrative plasmids. No plasmid is known which is self replicating in M. xanthus. Consequently, plasmids can only be maintained by integration into the chromosome. One way to achieve this is through the homologous recombination of a plasmid containing cloned M. xanthus sequences. This method has been used to create partial diploids (Shimkets et al. 1983). Integrative plasmids have also been constructed containing a promoterless lacZ gene. These can be used to measure transcription from a gene, rather like Tn5::lac.

Another use of homologous recombination is in site directed mutagenesis by gene replacement (Fig. 1-1). If there are M. xanthus sequences either side of an antibiotic resistance marker, then a double recombination is possible. This method has been used to mutate genes by transducing plasmids containing the same gene interrupted by Tn5. Double recombinants are recognisable by their lack of plasmid sequences (Fig 1-1). Site directed mutagenesis of the region coding for protein S has been carried out this way (Furuichi T. et al. 1985b).

Figure 1-1

Site-directed mutagenesis of *M. xanthus* by gene replacement

Possible outcomes of the introduction by transduction of *M. xanthus* DNA containing Tn5 sequences:

The cloned *M. xanthus* DNA and its homologue in the chromosome is shown as open boxes.

The transposon Tn5 is shown as a cross-hatched area

Thick lines denote other plasmid sequences.

Thin lines denote chromosomal sequences.

- a. A single recombination event between plasmid and chromosome results in the integration of the entire plasmid.
- b. A double recombination results in the integration of Tn5 without the accompanying plasmid. The site of integration is homologous to the site occupied within the plasmid. A mutant allele of the cloned gene will have been obtained.

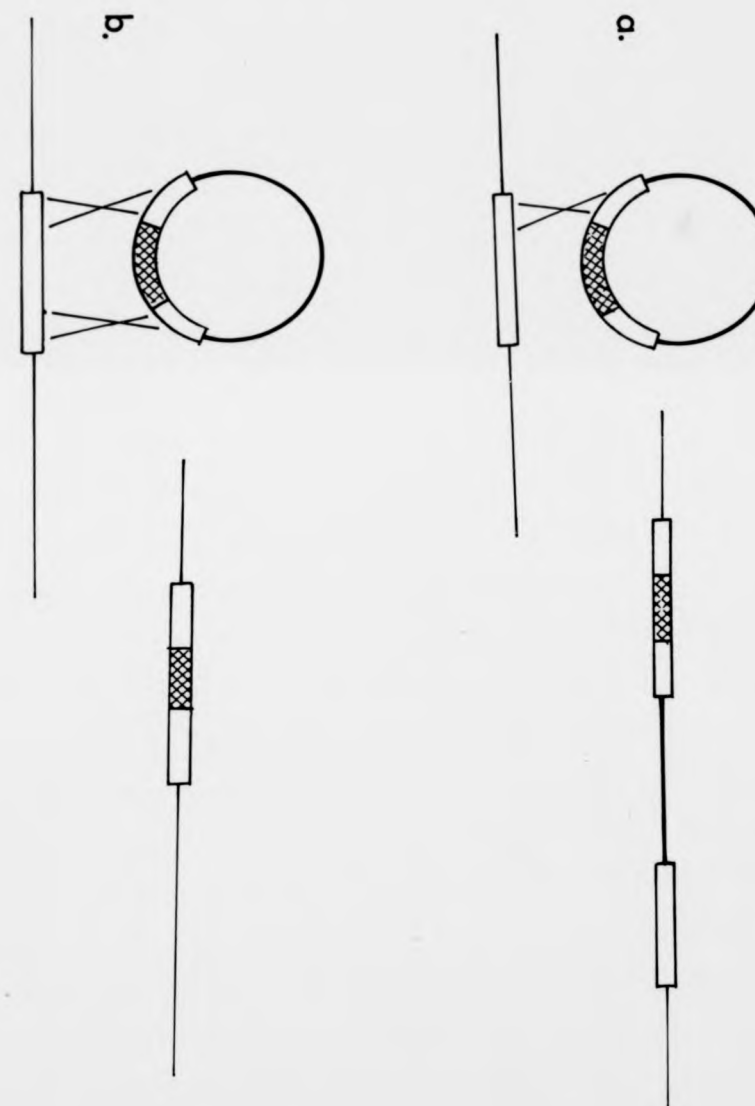


Fig. 1-1



### 1-3-5 Design of homology based promoter-probes

In order for a promoterless lacZ gene to be integrated by homologous recombination, the plasmid must contain M. xanthus DNA next to the lacZ. By having a unique restriction site upstream of the lacZ, any M. xanthus sequence can be chosen. Alternatively, a random library of chromosomal fragments can be cloned in the plasmid (Fig. 1-2). The resulting plasmid is packaged by infecting the host E. coli with P1 phage. The phage can package any DNA of a size between 45 and 105kb. Concatamers of the plasmid can be packaged instead and hence be transferred to M. xanthus. This process can be inefficient for small plasmids, and genes transduced in this manner may be in multiple copies. For this reason, plasmids to be packaged in P1 are now usually designed containing the cloned P1 incB region. Homologous recombination can then take place between single copy plasmids and the phage. A cointegrate of phage and plasmid is transferred during transduction (Shimkets L.J. et al., 1982)(Fig. 1-3). The two possible outcomes are shown in Fig. 1-4. In the first case, the cloned sequence contains a promoter. In this case, the promoter directs transcription of lacZ. The existing chromosomal copy of the gene remains intact. In the second instance, there is no promoter in the plasmid sequence. Here, a gene fusion is still produced but the functional copy of the gene is inactivated.

Fig 1-2

Construction of a gene library in a plasmid based promoter-probe

The BamHI site is upstream of the promoterless lacZ gene.

Fig. 1-2

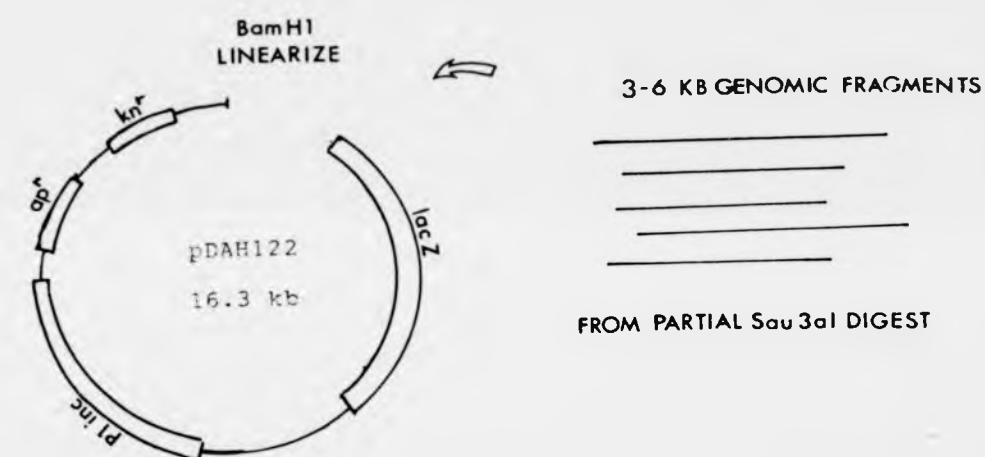


Fig. 1-3

Packaging of plasmid by P1 phage for transduction into M. xanthus

In E. coli a phage-plasmid cointegrate forms under combined selection for kanamycin and chloramphenicol resistance. The temperature is nonpermissive for lytic growth.

At permissive temperatures, phage are formed and these can transduce M. xanthus. The plasmid then excises from the phage genome and can integrate into the chromosome by homologous recombination.

Fig. 1-3

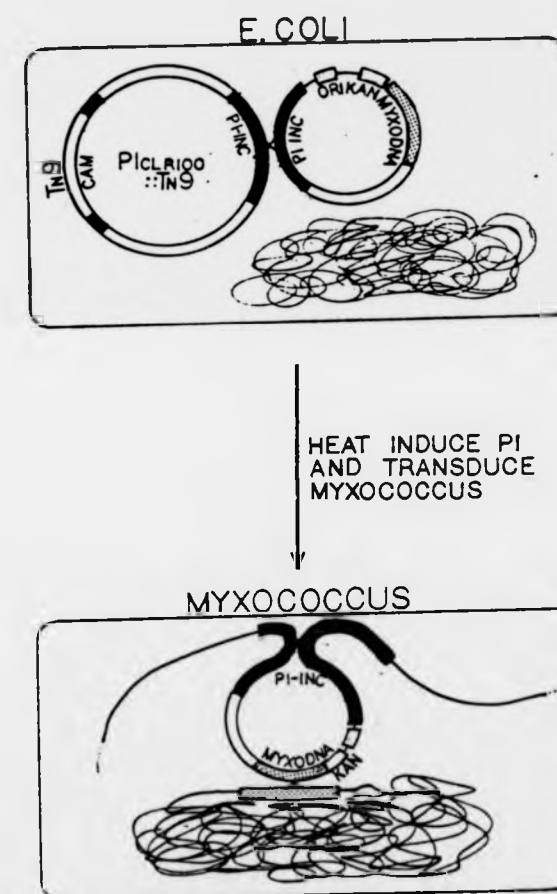


Fig. 1-4

The two possible outcomes of the integration of a plasmid based promoter-probe containing *M. xanthus* DNA. The integration is reversible.

Gene promoters are marked with the letter P with an arrow to show the direction of transcription.

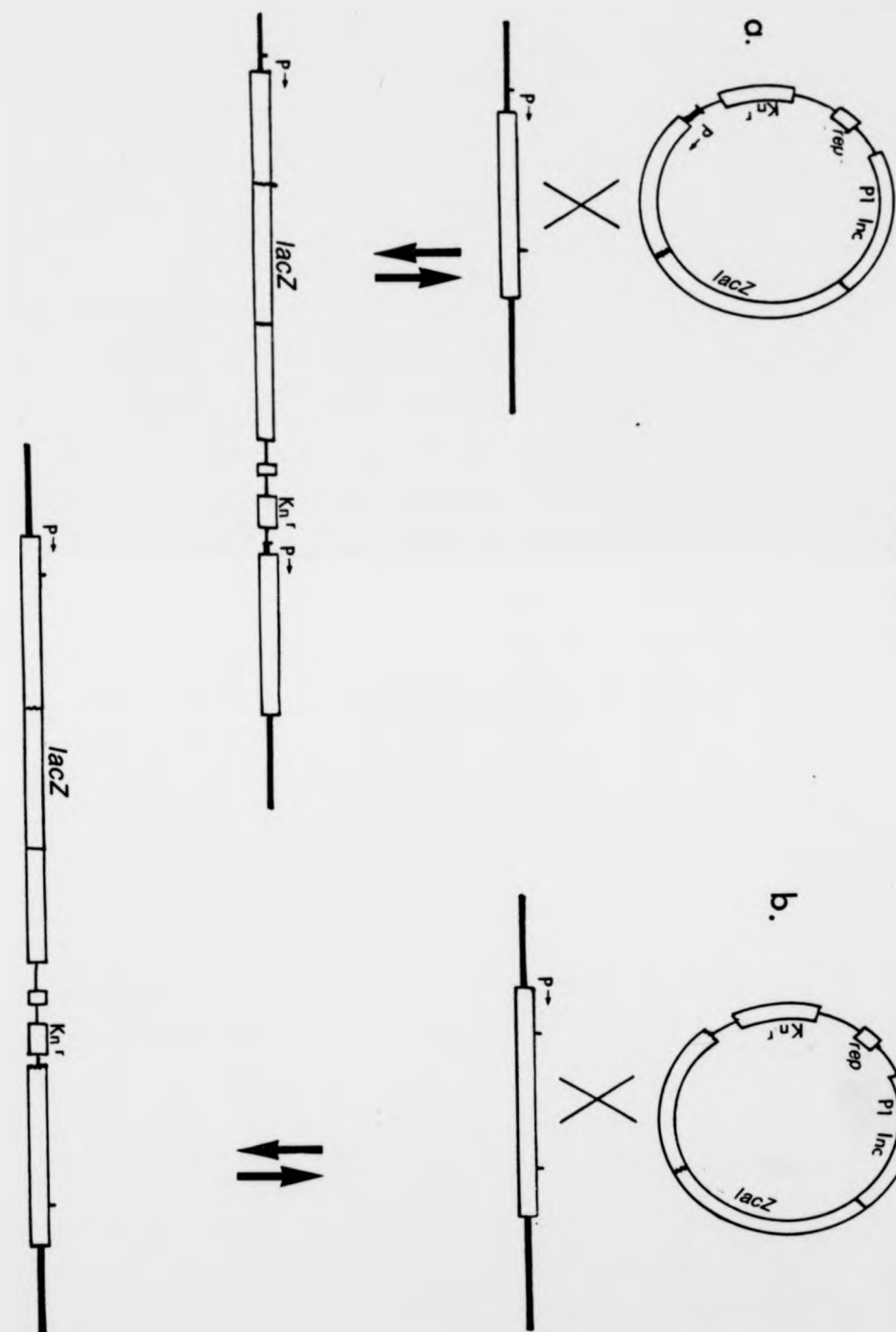
Thick lines denote *M. xanthus* sequences not within the cloned region.

Open boxes denote other plasmid sequences.

In a. There is a promoter in the cloned *M. xanthus* DNA. Once integrated into the chromosome, the promoter can direct transcription of the adjacent *lacZ* gene. The existing copy of the promoter and its corresponding operon remain intact.

In b. There is no promoter in the cloned region, as the cloned region is entirely within an operon. In this case, a gene fusion is formed. Furthermore the copy of the operon already present in the chromosome is truncated.

Fig. 1-4



### 1-3-6 Advantages of homology based promoter-probes

#### 1-3-6-1 The existing gene is not inactivated

Unlike a gene fusion which has been created by transposon insertion, a homologue of the fused gene remains intact. This happens as long as the cloned sequence contains a promoter. This is almost certain to be the case if the cloned sequence is several kilobases in length. This makes it possible to investigate the regulation of essential genes whose inactivation would give rise to a lethal mutation. Even with a nonessential gene, it may be important that gene regulation is investigated in a unmutated background. This would be an important consideration where a gene regulates its own transcription.

#### 1-3-6-2 Ease of cloning

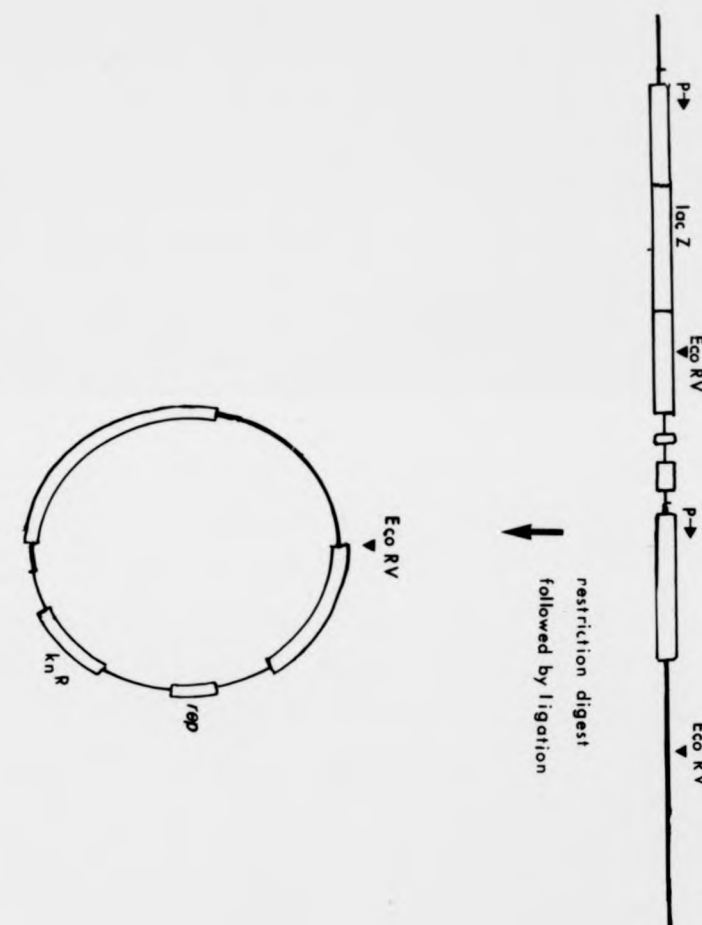
The process of homologous recombination is reversible. Consequently, a plasmid integrated by such means will undergo excision at a low frequency (Fig. 1-4), and a minority of cells in a culture will contain the plasmid as supercoiled circular DNA. These can be isolated on a caesium chloride gradient and amplified by transformation of E. coli. Such rapid cloning is especially valuable where a bulked library of chromosomal DNA was used in the transduction, a small number of clones of interest can then be isolated. The plasmid cloned

Fig. 1-5

Cloning of the gene for which a gene fusion has been obtained

A restriction enzyme is chosen which cuts within the plasmid but not within the *M. xanthus* DNA already cloned. The enzyme shown here is *EcoRV*. The enzyme is used to cut chromosomal DNA obtained from a strain containing the integrated plasmid. A ligation reaction is performed (in a large volume to favour circularisation of the fragments). A plasmid can be obtained from the fragment containing the plasmid replication origin. The plasmid should contain additional DNA sequences to those cloned in the gene fusion. These will be from downstream of the fusion point.

Fig. 1-5



in this way can be used to transduce other strains of M. xanthus, allowing the gene fusion to be investigated in different genetic backgrounds.

It is also possible to clone the intact copy of the gene. Using a restriction enzyme which cuts in the plasmid but not in the cloned gene (Fig. 1-5), a region corresponding to the gene fusion plus additional downstream sequences, can be cloned as a plasmid.

#### 1-3-6-3 Mutagenesis of the cloned genes

Once they are cloned, both the gene fusion and the intact gene can be studied by site-directed mutagenesis in E. coli followed by transduction into M. xanthus. In the case of the gene fusion, it should be possible to find the approximate position of the gene promoter under regulation, and possibly additional sequences required for correct regulation. The regulation of expression of Protein 5 has already been investigated this way (Downard J.S. et al. 1988).

The intact copy of the gene can likewise be mutagenised. If a selectable marker is placed in the gene, gene replacement is possible. It may be possible to define the function of the gene, if a mutant phenotype is created by this process.

#### 1-4 Genetics of Mixococcus development

##### 1-4-1 Introduction

Many developmental mutants have been isolated in M. xanthus. These may be specifically defective in sporulation. Others have motility defects recognisable in vegetative cells and so are unable to aggregate normally. Another category have normal motility but, in spite of this, are unable to undergo developmental aggregation. Finally, there are mutants which are defective in glycerol induced sporulation.

##### 1-4-2 Motility mutants

Mutants defective in motility are easily distinguishable by colony morphology. Nonmotile mutants form small, smooth edged colonies quite different from the spreading wild type colonies whose edges consist of streams of cells. Nonmotile mutants can regain motility, either by spontaneous reversion, or as a result of generalised transduction with Mx8 containing the wild type allele. With the exception of one mutant locus (mg1), two mutations are always required to render wild type cells completely nonmotile. It appears from this that there are two motility systems. With one of these, Social (S) motility, cells can glide but only when in close contact with one another. The other motility system, Adventurous (A), enables the cells to



move as individuals with little tendency to remain in contact. Transduction studies on the mutants (Hodgkin J. and Kaiser D. 1979ab) showed that H and S were both complex gene systems with at least 23 genes required for system H, and at least 10 for system S. The mgI locus required by both systems has since been identified as a single gene with two open reading frames (Stephens K. and Kaiser D. 1987). Certain mutants in both the H and S systems can be stimulated to move if they are in contact with a different motility mutant. Once initiated, the motility continues for up to an hour. This property of extracellular complementation is probably due to the existence of an intercellular signal for each motility system.

The mechanisms of motility, for which these genes are required, is largely unknown. However, the S motility system correlates with the presence of pili (also known as fimbriae) on the cells (Dworkin M. and Kaiser D. 1985). Mutants lacking S motility also lack pili. Stimulatable S mutants temporarily acquire pili when they are motile following extracellular complementation. Furthermore, the maximum distance from their neighbours at which H<sup>+</sup> cells can still move (3.5µm) corresponds to the length of the pili.

Other types of motility mutant have been identified which alter the periodicity of cell motility. These are the frizzy mutants (Blackhart B.D. and Zusman D.R. (1985)), named from their aberrant fruiting bodies. Normal cells reverse their direction of gliding every  $6.8 \pm 2.5$  minutes. Most frizzy mutants change direction once only several hours, although one frzD changes every  $2.2 \pm 0.3$  minutes and this leads to small, pinprick colonies. It appears that the wild type periodicity is essential for the formation of stable aggregates during

fruiting body formation.

To what extent are motility mutants capable of fruiting and sporulation? The complete elimination of motility as in an  $H^+S^-$  strain also eliminates the ability to form fruiting bodies. Most  $H^+S^+$  mutants can form normal, if undersized, fruiting bodies. By contrast,  $H^+S^-$  mutants are unable to move as aggregates and many are unable to form fruiting bodies. However, mutants unable to form fruiting bodies, even those lacking both motility systems, are still able to form spores (Hodgkin J. and Kaiser O. 1979b).

#### 1-4-3 Sporulation mutants

Certain mutants affect the sporulation mechanism. This may or may not affect aggregation and fruiting body formation as well. Many sporulation mutants can be rescued by extracellular complementation. Pairs of mutants, unable to sporulate on their own, can sporulate as mixtures. The spores only give rise to the original mutants, indicating that no genetic complementation has taken place. Four complementation groups have been identified, representing at least 57 mutants (Hagen D.C. *et al.* 1978). Two groups, asg and bsg, contain many genes from different loci. Group csg maps to one locus and has now been identified as a single gene (Shimkets L.J. and Asher S.J. 1988). Group dsg mutants map to not more than two loci. The phenomenon of extracellular complementation could be explained by either intercellular signalling, or by metabolic crossfeeding. The former seems more likely because there are few complementation groups compared with the number

of genes. Moreover, the mutants do not have any auxotrophic requirements in the vegetative state. The four classes of mutant appear to be blocked at different stages as judged by their appearance and by biochemical markers of sporulation (Table 1-2). Another characteristic of each complementation group is the pore size through which the extracellular signalling can take place (Table 1-2).

In the case of csg mutants, sporulation can also be restored by adding peptidoglycan monomers. Diaminopimelate, D-alanine, N-acetylglucosamine and N-acetylmuramic acid can all rescue sporulation to some extent. All four together can act synergistically, restoring normal levels of sporulation. The resulting spores have normal structure and protein composition (Shimkets L.J. and Kaiser D. 1982).

#### 1-4-4 Aggregation mutants

The process of aggregation into fruiting bodies, as distinct from general motility, can also be affected in certain mutants. Some of these are also defective in sporulation and could equally be classified in the previous section. Others are still capable of sporulation in the absence of fruiting bodies (Morrison C.E. and Zusman D.R. 1979). This is further evidence for branched pathways for aggregation and sporulation. Extracellular complementation is observed with some aggregation mutants (Shimkets L.J. 1986). There are two complementation classes, dsp and igl. However, both of these have motility defects as well as being affected in aggregation. A gene cluster not in an extracellular

TABLE 1-2

Properties of the four classes of sporulation mutant which can be grouped by their properties of extracellular complementation

Class of strain:	wild type	<u>asg</u>	<u>bsg</u>	<u>csq</u>	<u>dsg</u>
HBHn produced	+	-	+	+	-
Protein S produced	+	delayed	+	+	+
Sensitive to phenylalanine starvation	+	-	+	+	+

Membrane pore size across which sporulation can be restored by extracellular complementation

	<u>asg</u>	<u>bsg</u>	<u>csq</u>	<u>dsg</u>
0.2mm polycarbonate	+	+	+	+
50000 dalton dialysis membrane	-	+	+	+
10000-12000 dalton dialysis membrane	-	+	+	-
3000 dalton dialysis membrane	-	-	+	-

complementation group has also been characterised (Torti S. and Zusman D.R. 1981). These are the rough mutants. These are unable to begin aggregation. However sporulation is still possible.

#### 1-4-5 Glycerol resistant mutants

A minority of mutants isolated for their inability to sporulate under starvation conditions, are also unable to sporulate when glycerol is added to cells in liquid medium (Morrison C.E. and Zusman D.R. 1979). Where mutants are isolated for their inability to sporulate in glycerol, most of these are still able to undergo normal developmental sporulation (Burchard R.P. and Parish J.H. 1975). There are also mutants which are noninducible by glycerol but which still sporulate when phenethyl alcohol is used instead.

#### 1-4-6 Reverse genetics

If a protein of interest is available in sufficient quantity to be sequenced, it is then possible to construct an oligonucleotide probe to detect the corresponding gene in a chromosomal library. The region coding for protein S has been cloned this way (Inouye S. et al. 1983a). The oligonucleotide probe hybridised to Southern blots to give two bands with some restriction digests but only one band with others. This suggested that the region contained two copies of the same gene. Sequencing of the region (Inouye S. et al. 1983b)

revealed two genes with 88% homology separated by a 1.4 kb spacer. The further downstream of the two genes (gene tps) codes for protein S. The upstream homologue (ops) codes for a protein of unknown function, found within the spore (Teintze M. et al. 1988). Deletion mutants of either gene can still sporulate normally. If both genes are deleted along with the spacer, then sporulation is delayed by 12 hours (Furuichi T. et al. 1985b).

A similar approach has been used to clone the gene coding for MBHA, a protein for which the amino acid sequence is known (Romeo J.M. and Zusman D.R. 1987). Mutants have then been constructed which are unable to produce the protein. Again, sporulation still occurs but is delayed. The delay compared with wild type is most marked under suboptimal sporulation conditions such as low magnesium or low cell density. The protein may have a more important role in the natural environment than under laboratory conditions.

#### 1-4-7 Gene fusions

The use of promoterless lacZ genes, whether as In5::lac genes or as homology based insertions, provides a means to measure expression of a particular gene. For example, gene fusions have been made of the ops gene (Downard J.S. et al. 1984) and the tps gene (Downard J.S. and Zusman D.R. 1985). Gene fusions were constructed as plasmids in vitro. In both cases, when transduced into Myxococcus, developmentally regulated expression of  $\beta$ -galactosidase was observed. The ops gene fusions are transcribed later than tps and, unlike tps,

are transcribed in spores induced by glycerol.

Another approach has been to make random fusions throughout the chromosome using Tn5::lac (Kroos L. et al. 1986). Among 2374 transductants, 36 showed some increase in expression under sporulating conditions over that under vegetative conditions. These represented at least 29 different transcription units. However, only seven of these showed any developmental abnormalities. This would suggest that most developmentally induced genes are not absolutely required for development, at least not under laboratory conditions. Alternatively, it could mean that there are alternative developmental pathways.

Gene fusions which are developmentally regulated, are useful developmental markers. Gene fusions characterised in a wild type background (Kroos L. et al. 1986) have been transferred to asg backgrounds (Kuspa et al. 1986) and to bsg and csg backgrounds (Kroos L. and Kaiser D. 1987). The findings suggest that there is a sequence of intercellular signals (Fig. 1-6). Group C is required at a later stage than the requirements for groups A and B. However, not all the dependencies upon B or C are clear cut. Group B must be active from the outset of development in order to achieve full expression of any developmental gene, although an absolute requirement for B does not appear until much later. Similarly, there is a partial requirement for Group C at an earlier stage of development. Only later, is expression of that complementation group essential for development.



36



## CHAPTER 2

### Materials and methods

## 2-1 Strains used

2-1-1 Escherichia coli K12

strain:	genotype:	reference:	source:
C600	<u>hsdR</u> <sup>-</sup> , <u>hsdM</u> <sup>+</sup> , <u>lacY</u> , <u>conA21</u> <u>leuB6</u> , <u>thr</u> <sup>-1</sup> , <u>supE44</u> , F <sup>-</sup>		D. A. Hodgson
NC1061	<u>hsdR</u> <sup>-</sup> , <u>galU</u> <sup>-</sup> , <u>galK</u> <sup>-</sup> , <u>str<sup>r</sup></u> $\Delta$ ( <u>ara</u> , <u>leu</u> )7697, $\Delta$ ( <u>lacI</u> P O Z Y)	Casadaban and Cohen (1980)	D. A. Hodgson
RB308	F <sup>+</sup> , <u>deoC</u> , <u>lacY</u> , <u>thyA</u>	Jones and Holland (1984)	D. Gill
HB101	<u>hsdR</u> <sup>-</sup> , <u>hsdM</u> <sup>-</sup> , <u>recA13</u> , F <sup>-</sup> <u>ara-14</u> , <u>proA2</u> , <u>lacY1</u> , <u>lacZ4</u> <u>rpsL20</u> , <u>xyl-5</u> , <u>purE</u> , <u>galK2</u> , <u>mtl-1</u> , <u>strA</u> , <u>leuB6</u> , <u>thr-1</u> , <u>supE44</u>	Maniatis et al. (1982)	D. Gill
LE392	<u>hsdR</u> <sup>-</sup> , <u>supE44</u> , <u>supF38</u> , F <sup>-</sup> , <u>lacY1</u> , <u>galK2</u> , <u>galT22</u> , <u>metB1</u> , <u>trpR55</u>	de Bruijn, Lupski (1984)	D. Gill
CSH26- DF6	<u>thi</u> , <u>rpsL</u> , <u>sup</u> <sup>o</sup> , F <sup>-</sup> <u>recA-cr</u> , $\Delta$ ( <u>lac-pro</u> ), $\Delta$ ( <u>recA-srl</u> )F6	Jones, Holland (1984)	D. Gill

2-1-2 E. coli phages

strain:	genotype:	reference:	source:
P1::Tn9 ts	P1::Tn9 <u>cln-100</u> <u>Tn973cm<sup>r</sup></u>	Rosner (1972)	D. Hodgson
$\lambda$ ::Tn5	$\lambda$ <u>c1857</u> , <u>b221</u> , <u>rex</u> ::Tn5 <u>P<sub>am</sub>80</u>	de Bruijn and Lupski (1984)	D. Hodgson
P1::Tn5-132	P1::Tn5-132 <u>cln100</u>	Avery and Kaiser (1983)	D. Hodgson

2-1-3 Existing Myxococcus xanthus strains

strain:	Phenotype:	reference:	source:
DK101	partial Sgl	Hodgkin, Kaiser (1979a)	D. A. Hodgson
DK1622	Reconstructed wild type	Kaiser (1979)	D. A. Hodgson
DK306	Mgl	Hodgkin, Kaiser (1979a)	D. Kaiser
DK5057 <u>asgA476</u> <u>Ω-4650</u>	Asg. Kn <sup>r</sup>	Hagen <u>et al.</u> (1978)	D. Kaiser
DK4726 <u>asgB480</u> <u>Ω-4678</u>	Asg. Kn <sup>r</sup>	Hagen <u>et al.</u> (1978)	D. Kaiser
M252 <u>bsgA301</u>	Bsg	Gill <u>et al.</u> (1987)	R. Gill
LS202 <u>csg-741</u>	Csg	Shimkets, Asher (1987)	L. Shimkets
DK3621 <u>dsg-439</u> <u>Ω-1867</u>	Dsg	Hagen <u>et al.</u> (1978)	D. Kaiser

2-1-4 M. xanthus strains prepared during the course of this work

Strain:	Derivation:	Phenotype:	See also in text:
UWM1	Gene library	kn <sup>r</sup>	Section 3-3
UWM2	pDAH122::M. xanthus		
UWM3	chromosomal DNA		
UWM4	transduced into		
UWM5	DK101		
A45	Gene library	kn <sup>r</sup>	Section 3-4
A48	pDAH122::M. xanthus		
D32	chromosomal DNA.		
EB	transduced into		
	DK1622		
V-1-3	Gene library	kn <sup>r</sup>	Section 3-4
S-24	pDAH283::M. xanthus		
V-1-20	chromosomal DNA		
V-3-2	transduced into		
	DK1622		
DK1000	Spontaneous mutant	kn <sup>s</sup>	Section 3-6
	appearing originally		
	in strain UWM5	aggregation/	
	then cured of	motility	
	integrated plasmid	defective	

2-2 Plasmids

2-2-1 Existing plasmids

Name:	Comments:	Phenotype:	Reference:	Source:
pDAH122	Integrative <u>lacZ</u> promoter probe	ap <sup>r</sup> kn <sup>r</sup>	Hodgson (unpublished)	D. A. Hodgson
pDAH283	Improved version of pDAH122	ap <sup>r</sup> kn <sup>r</sup>	Hodgson (unpublished)	D. A. Hodgson
ColE1::Tn5-132	Source of tetracycline resistance gene	ap <sup>r</sup> tc <sup>r</sup>	Berg <u>et al.</u> (1981)	D. A. Hodgson
pHP45Ω	Source of omega fragment	ap <sup>r</sup> Sm <sup>r</sup> /Spc <sup>r</sup>	Prentki and Krisch (1984)	D. Gill

2-2-2 Plasmids prepared in the course of this work  
(continued)

Name:	Derivation:	Phenotype:	See also in text:
PUWM4'tc	PUWM4' with the <u>tc</u> gene from <u>colE1::Tn5</u> inserted at the <u>HindIII</u> site	<u>tc</u> <sup>r</sup>	Section 6-3
PUWM5':::Tn5(-200) PUWM5':::Tn5(-800) PUWM5':::Tn5(-1300) PUWM5':::Tn5(-2000)	Transposon inserts in PUWM5'D23 with number to show distance of insert from fusion point	<u>ap</u> <sup>r</sup> <u>kn</u> <sup>r</sup>	Section 6-5 Fig. 6-5
PUWM5':::Tn5(-1300)	Transposon insert in PUWM5 with number to show distance of insert from fusion point	<u>ap</u> <sup>r</sup> <u>kn</u> <sup>r</sup>	Section 7-4-1
PUWM5D	Plasmid PUWM5 with 9.5kb deleted	<u>kn</u> <sup>r</sup>	Section 7-3-1
PUWM4D	Plasmid PUWM4 with 9.5kb deleted	<u>kn</u> <sup>r</sup>	Section 7-3-1
PUWM4D::Y6(-5000) PUWM4D::Y6(-3100) PUWM4D::Y6(-1200) PUWM4D::Y6(-200)	Transposon insert with number to show distance of insert from fusion point	<u>kn</u> <sup>r</sup>	Section 7-3-1
PUWM5::Ω	Plasmid PUWM5D with the Ω fragment inserted in the <u>StuI</u> site	<u>kn</u> <sup>r</sup> <u>Spc</u> <sup>r</sup> / <u>Sm</u> <sup>r</sup>	Section 7-5-1

## 2-2-3 Genes characterised in this work; their nomenclature

Name:	Description:	Occurrence:
<u>isg-1</u> <u>=isgA1</u>	Inducible sporulation and glycerol-1	Cloned in plasmids pUWM4', pUWM4'tc and derivatives
<u>isg-1&gt;lacZ</u> <u>=isgA1&gt;lacZ</u>	Gene fusion of <u>isg-1</u> promoter with <u>lacZ</u>	Occurs in plasmids pUWM4, pUWM4D and derivatives
<u>isg-2</u> <u>=isgB2</u>	Inducible sporulation and glycerol-2	Partially cloned in plasmids pUWM5, pUWM5', pUWM5'023 pUWM5D and derivatives Restriction map of entire region has been prepared (Fig. 5-4)
<u>isg-2&gt;lacZ</u> <u>=isgB2&gt;lacZ</u>	Gene fusion of <u>isg-2</u> promoter with <u>lacZ</u>	Occurs in plasmids pUWM5, pUWM5D and derivatives

2-2-4 Tn5 inserts in M. xanthus

These were prepared by transduction with a plasmid containing a Tn5 insert and contain a copy of Tn5 at the homologous location in the chromosome to that in the plasmid. The plasmid location has been checked by Southern analysis (Table 6-6).

Insert strain:	Plasmid used in transduction:
DK1622[ $\Omega$ S-2000]	pUWM5': :Tn5(2000)
DK1622[ $\Omega$ S-1300]	pUWM5': :Tn5(1300)
DK1622[ $\Omega$ S-800]	pUWM5': :Tn5(800)
DK1622[ $\Omega$ S-200]	pUWM5': :Tn5(200)

## 2-3 Chemicals, media and antibiotics

## 2-3-1 Chemicals

Chemicals were obtained either from Fisons, May and Baker or Sigma. Restriction enzymes and DNA modification enzymes were obtained from Amersham International, Bethesda Research Laboratories or Boehringer Mannheim. Radiochemicals were obtained from Amersham International.

## 2-3-2 Buffers

Z-buffer (used in  $\beta$ -galactosidase and protein assays)

0.06M  $\text{Na}_2\text{HPO}_4$   
0.04M  $\text{NaH}_2\text{PO}_4$   
0.01M KCl  
0.001M  $\text{MgSO}_4$

TBE (used for agarose gel electrophoresis)

0.089M Tris base  
0.089M boric acid  
2.5mM EDTA

TE (used for dissolving DNA)

10mM Tris.Cl pH8.0  
1mM EDTA

SSC (for Southern blotting and hybridisation)

SSC is used at a variety of concentrations. 20x SSC is:

3M NaCl  
0.3M sodium citrate

## 2-3-3 Media

LB (for E. coli cultures)

Bacto tryptone (Difco)	10g
Yeast extract (Difco)	5g
NaCl	5g
H <sub>2</sub> O	1 litre

LB agar is made as above but with 15g Bacto agar per litre for normal agar and 7g per litre for soft agar.

SOB (for transformations of E. coli)

Bacto tryptone (Difco)	20g
Yeast extract (Difco)	5g
1M NaCl	10ml
1M KCl	2.5ml
1M MgCl <sub>2</sub>	10ml (filter sterilized and added after autoclaving)
H <sub>2</sub> O	1 litre

ODH (for  $\lambda$  preparation)

Bacto tryptone (Difco)	20g
NaCl	5g
1M MgCl <sub>2</sub>	10ml (added after autoclaving)
H <sub>2</sub> O	1 litre
agar	10g
	(3.5g for soft agar)

Obi T (for P1 phage preparation)

Bacto tryptone	20g
NaCl	5g
H <sub>2</sub> O	1 litre

(the following are added after autoclaving)

1M MgSO <sub>4</sub>	10ml
1M CaCl <sub>2</sub>	1ml
0.5% thiamine	1ml
30% glucose	10ml

CTTE (Myxococcus liquid medium)

Casitone (Difco)	10g
yeast extract (Difco)	2g
1M Tris.HCl pH8	10ml
1M MgSO <sub>4</sub>	10ml
H <sub>2</sub> O	1 litre



DCY (Myxococcus solid medium)

Casitone (Difco)	20g
yeast extract (Difco)	2g
1M Tris.HCl pH8	10ml
1M MgSO <sub>4</sub>	8ml
H <sub>2</sub> O	1 litre
agar	15g
(7g for soft agar)	

TM (liquid) (for resuspending Myxococcus cells)

1M Tris.HCl pH8	10ml
1M MgSO <sub>4</sub>	8ml
H <sub>2</sub> O	1 litre

TM (solid) (for starvation induced sporulation)  
as above but with 15g noble agarCF agar (for starvation induced sporulation) (Hagen D.C.  
et al. 1978)

Casitone (Difco)	0.15g
1M Tris.Cl pH8	10ml
1M MgSO <sub>4</sub>	8ml
1M K <sub>2</sub> HPO <sub>4</sub> pH 7.4	1ml
H <sub>2</sub> O	1 litre

## (after autoclaving add)

4% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5ml
20% sodium pyruvate	5ml
20% sodium citrate	10ml

A1 medium (Myxococcus minimal medium) (Bretcher A.P. and  
Kaiser D. 1978)

2M Tris.HCl pH7.6	5ml
1M MgSO <sub>4</sub>	0.8ml
1M K <sub>2</sub> HPO <sub>4</sub> pH7.6	1ml
1M CaCl <sub>2</sub>	0.01ml
100mM FeCl <sub>3</sub>	0.1ml
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5g
leucine	50mg
isoleucine	100mg
phenylalanine	100mg
valine	100mg
methionine	10mg
H <sub>2</sub> O	920ml
Noble agar	15g

## (added after autoclaving, all filter sterilized)

10% potassium aspartate	50ml
20% sodium pyruvate	20ml
50mg/ml asparagine	2ml
125mg/ml spermidine	1ml
1mg/ml vitamin B <sub>12</sub>	1ml

## 2-3-4 Antibiotics

The following antibiotics were used. Some could be stored as stock solutions, others had to be freshly dissolved every time. Antibiotics were dissolved in water unless otherwise stated.

Antibiotic:	Stock solution:
Ampicillin (ap)	100mg/ml stored as aliquots at 4°C
Cephapirin (cp)	100mg/ml made freshly as required
Chloramphenicol (cm)	20mg/ml stored dissolved in absolute ethanol at -20°C
Kanamycin (kn)	100mg/ml stored at 4°C
Oxytetracycline (oc)	10mg/ml in 95% ethanol, made freshly as required
Spectinomycin (spc)	20mg/ml stored at 4°C
Streptomycin (sm)	100mg/ml stored at 4°C
Tetracycline (tc)	10mg/ml stored dissolved in absolute ethanol at -20°C

## 2-4 Growth and maintenance of cultures

E. coli was grown at 37°C in LB broth or on LB agar. Liquid cultures were shaken at 280rpm in an orbital shaker. E. coli could be maintained on plates at 4°C for up to 2 months. For longer term storage 10% glycerol was added to samples of culture and these were stored at -70°C. Cells from plates were also stored as stabs in soft agar.

M. xanthus was grown at 33°C in CTTYE broth or on DCY agar. Liquid cultures were shaken at 240rpm in an orbital shaker. M. xanthus plates could be stored at 17°C for 2-4 weeks. For longer term storage they were stored in 10% DMSO at -70°C or on slopes of H1 agar.

## 2-5 Preparation of phage lysate

### 2-5-1 P1 lysates

A dilution series of phage was made from an existing phage lysate. Dilutions from  $10^{-2}$  to  $10^{-7}$  were made in Dbl T. E. coli strain C600 was grown in LB + 10mM MgSO<sub>4</sub> to a density of  $A_{600}=0.3$ . To 0.1ml of each phage dilution was added 0.1ml of culture. The phage was allowed to adsorb for 20 minutes and then 3.5ml of molten Dbl T soft agar was added and the mixture poured onto a plate of Dbl T agar.

After 16 hours at 37°C, plaques could be counted. A phage

dilution was made from the original lysate calculated to give almost confluent plaques. Phage lysates were prepared from these. Phage could then be eluted from the plates containing confluent plaques, by pouring 5ml of Dbl T broth onto each plate and allowing it to stand for 2 hours at room temperature, or overnight at 4°C. The liquid was removed from the plates, briefly vortexed with chloroform and then centrifuged at 5000rpm at 4°C in an MSE Chillspin. The supernatant containing the phage was removed and could be stored at 4°C over chloroform.

#### 2-5-2 Lambda lysates

Phage lysates were prepared in the strain LE392. A small number of phage plaques (two to five) were resuspended in phage buffer. Samples of this: 10µl, 50µl, 100µl and 200µl were added to separate aliquots of overnight cultures. After 20 minutes, 3ml of molten DDA soft agar was added to each aliquot, and each mixture overlaid onto a freshly made DDA plate. After 16 hours at 37°C, the plate showing confluent lysis was taken, the soft agar removed using a glass spreader and the remaining agar washed with 3ml phage buffer, which was then pooled with the soft agar in a glass universal. This was vortexed with 0.5ml of chloroform for 15 minutes and then centrifuged in an MSE chillspin for 15 minutes at 5000rpm at 4°C. The supernatant phage lysate was then removed. It could be stored over chloroform at 4°C.

2-6 P1 mediated transduction of M. xanthus

## 2-6-1 Packaging of plasmid into P1

The phage used for packaging was P1::Tn9 clr100. The plasmid was maintained in a rec<sup>+</sup> strain of E. coli such as MC1061. This strain was grown in LB containing 10mM HgCl<sub>2</sub> and with appropriate antibiotic selection. When the culture reached a density of  $A_{550}=0.21$  the phage were added to 0.1ml of culture at a M.O.I. of 10 and allowed to adsorb for 20 minutes at room temperature. The mixture was then diluted into LB + Mg<sup>2+</sup> + antibiotic. This time, 12.5µg/ml chloramphenicol was added as well, to select for phage-plasmid cointegrates. The culture was shaken overnight at 30°C. The next day, a dilution was made and allowed to grow at 30°C to a density of  $A_{550}=0.21$ . Lytic growth was then induced by transferring the culture to a shaking water bath at 42°C for 35 minutes followed by 37°C for 2 hours. Cell lysis was apparent after this treatment. To the lysate was added 0.1ml of chloroform and shaking continued at 37°C for 15 minutes. After this, cell debris was removed by spinning in a universal for 15 minutes in an MSE Chillspin centrifuge at 5000rpm at 4°C for 10 minutes. The lysate could be stored over chloroform at 4°C for several weeks.

## 2-6-2 P1 transduction of M. xanthus

The P1 phage containing packaged plasmid or other insert was used to transduce Myxococcus to kanamycin or oxytetracycline resistance. Dilutions of phage were made in TM containing 50, 100 and 200  $\mu$ l of lysate to a total volume of 0.4 ml. To each of these was added 0.1 ml of 50 mM CaCl<sub>2</sub>, followed by 0.5 ml of Myxococcus culture ( $A_{660}$ =0.8-1.0). The phage was left to adsorb for 40 minutes at room temperature. Then 3.5 ml of molten DCY soft agar was added to each sample and overlaid on an agar plate containing 40  $\mu$ g/ml kanamycin or 10  $\mu$ g/ml oxytetracycline and the plate incubated at 33°C for 3-6 days for colonies to appear.

In the case of kanamycin selection, the yield of colonies could be increased by overlaying onto DCY + 25  $\mu$ g/ml kanamycin, and then increasing the total concentration to 70  $\mu$ g/ml kanamycin the next day, by adding the antibiotic in a second soft agar overlay. For selection for tetracycline resistance, oxytetracycline was used as it has a greater window for selection than tetracycline.

## 2-7 In5 mutagenesis of plasmids in E. coli

A lambda phage  $\lambda::\text{In5 } Q_{\text{am}}P_{\text{am}}$  is unable to undergo lytic or lysogenic development in nonsuppressing E. coli strains. Consequently, it is able to act as a suicide vector for introducing In5 into cells. The host background containing the

plasmid was MC1061. Overnight cultures were grown in 10ml LB with antibiotic selection for the plasmid and with 10mM  $\text{MgCl}_2$  and 0.2% maltose. The culture was spun down in a universal at room temperature in an MSE floor centrifuge at 5000rpm and resuspended in 1ml of LB + 10mM  $\text{MgCl}_2$ . To the suspension was added phage lysate containing  $10^8$  pfu. The mixture was incubated at 30°C for 2 hours to allow transduction and transposition to take place. The mixture was then plated out in varying dilutions, at a final volume of 0.1ml, onto LB + 50µg/ml kanamycin to select for Tn5. The resulting transductants were then picked onto LB plates containing kanamycin at 300µg/ml, and incubated overnight. Transductants with Tn5 integrated into the plasmid, grew vigorously even at this antibiotic concentration. By contrast, transductants arising through chromosomal integration grew only poorly.

#### 2-8 gamma-delta mutagenesis in E. coli

The method for mutagenesis, mediated by transposon gamma-delta carried on the conjugative F plasmid, has been described by Guyer A.S. (1983).

A plasmid to be mutagenised with gamma-delta was first introduced into the F<sup>+</sup> strain RB308 by transformation (see section 2-17). This was allowed to conjugate with a recipient strain MC1061, CSH26 or HB101. Donor and recipient strains were patched onto LB plates with antibiotic selection where appropriate. After 6-10 hours at 37°C, the cells were scraped off and mixed into a single patch on a third LB plate without

antibiotic. After 3-4 hours at 37°C to allow conjugation to occur, the cells were spread onto LB plates containing the appropriate antibiotic to select for the plasmid, and streptomycin to select against the donor strain. The plates were incubated overnight at 37°C to obtain colonies of transconjugants.

#### 2-3 Testing for sporulation in M. xanthus

Samples were examined for spores under the phase-contrast microscope. The spherical spores were readily distinguishable from the rod shaped vegetative cells. Furthermore, spores obtained from starved cells and some spores obtained by glycerol induction in liquid medium appeared bright under phase contrast.

It was sometimes necessary to test whether spores once formed were viable. Spores were incubated at 50°C for 2 hours to kill any remaining vegetative cells. A sample of vegetative cells was tested at the same time as control. The spores were then streaked onto a DCY plate which was examined for colonies after incubation for five days. Alternatively, where spores had formed under starvation conditions on a TM plate, the plate was incubated at 50°C for 2 hours. Then DCY soft agar was overlaid on the plate to allow viable spores to germinate and form visible colonies.



#### 2-10 Induction of sporulation by starvation

Colonies were sometimes picked directly onto a sporulation medium: either TM or CF. However, in order to obtain material in larger and more reproducible quantities it was more usual to spot cell suspensions onto plates. Dense cultures were either spun down in 2ml Eppendorf tubes in a microfuge or in universals at 5000rpm in a MSE benchtop centrifuge. The cells were resuspended in a smaller volume (typically 1/10) of TM. The suspension was then spotted onto dried agar plates in 10 or 20 $\mu$ l aliquots and the liquid allowed to absorb. Fruiting bodies and spores were apparent after several days incubation.

#### 2-11 Chemical induction of sporulation

Liquid cultures of M. xanthus were induced to sporulate by adding one of three chemicals: glycerol, dimethyl sulphoxide (DMSO) or phenethyl alcohol (PEA). Liquid cultures were grown in CTTYE at 33°C to a density of  $A_{600}=0.3$ . Densities in excess of this were found to sporulate poorly or not at all. Glycerol was added as a 50% solution in water to a final glycerol concentration of 0.7M. DMSO was added to a final concentration of 0.7M. PEA was added to a final concentration of 0.017M. The culture was then kept in shaking culture in CTTYE to allow spores to form.

## 2-12 Sonication of M. xanthus samples

Samples on IM plates were scraped off the agar using a coverslip, and suspended in 0.5ml of Z buffer (Section 2-4-3) in a 2ml Eppendorf tube. Samples in liquid medium were transferred to 2ml Eppendorf tubes and spun down in a microfuge. As the liquid medium was usually CITE which would affect protein assays, the supernatant was removed as completely as possible. The pellet was resuspended in 1ml of IM. This was spun down and the supernatant removed as before. The pellet was then resuspended in 0.5ml of Z buffer. Samples were stored in 2ml Eppendorf tubes at -20°C prior to sonication. These were thawed at room temperature and kept on ice until sonication. Sonication was carried out in two ways. An MSE sonicator was used with a 3mm probe. To aid spore breakage, 0.10-0.11mm glass beads (B. Braun Melsungen AG) were added (0.2-0.3ml volume). The tube was cooled on dry ice while the tube was sonicated 15s at a time with 15s intervals for cooling. This was continued for 6 minutes to allow adequate spore breakage. Needless to say, the fragile vegetative cells were also disrupted.

A second method was to use a cup horn sonicator (W380 sonicator with a 4318 cup horn, (Heat Systems Ultrasonics Inc.)). This easily disrupted vegetative cells without affecting spores. Up to eight tubes could be sonicated simultaneously. This was useful for sonicating samples known to contain only vegetative cells. Furthermore, it enabled separate vegetative cell and spore fractions to be taken from

a single sample. A sample was sonicated first in the cup-horn sonicator for one minute or until the samples containing only vegetative material had become clear. Samples with spores remained turbid. The spores were spun down in a microfuge. A white pellet was obtained containing only mature spores. The supernatant was removed and kept as the vegetative fraction. The spores were resuspended in 0.5ml of Z-buffer before glass spheres were added and the sample sonicated using the probe sonicator, as already described for breaking spores. Sonicated samples were kept on ice until assayed the same day for both  $\beta$ -galactosidase and protein.

#### 2-13 Protein assay

Sonicates were assayed for protein using the Biorad Coomassie blue micro assay (Bradford M.M. 1976). The buffer used in the assay was the same Z-buffer used in the  $\beta$ -galactosidase assay. Varying dilutions of bovine serum albumin were used as a standard.

#### 2-14 $\beta$ -galactosidase detection and assays

The enzyme  $\beta$ -galactosidase was detected in situ, using either the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal) (Maniatis T. et al. 1982) or the fluorogenic substrate 4-methyl umbelliferyl galactoside (4-MUG) (Youngman P. et al. 1985). X-gal was added to agar media at 40 $\mu$ g/ml. TM medium or CF medium was used to provide

sporulation conditions. M1 medium was used to provide conditions for vegetative growth.

A different technique was used where 4-MUG was used to detect  $\beta$ -galactosidase. Instead of being added to plates, it was dissolved in DMSO to a concentration of 2mg/ml and sprayed onto sporulating and vegetative cells on plates. The plates were left for 10-20 minutes and then placed on a long-wave UV transilluminator in order to visualise the blue fluorescence of 4-methyl umbelliferone released by hydrolysis of 4-MUG.

Quantitative assays were carried out on cell or spore sonicates using ortho-nitrophenyl galactoside (ONPG). The method used was that of Miller J.H. (1972), with the following exceptions. Firstly, no  $\beta$ -mercaptoethanol was used in the Z-buffer. Secondly, owing to the yellow colour of M. xanthus being similar to that of the ONP released in the assay, care had to be taken to eliminate this source of error. This was done either by ensuring that the sample was diluted to a point where its colour before assay was not apparent, or else by having a separate sample blank consisting of a sonicate with buffer added which contained no ONPG.

Units of enzyme were defined in terms of nanomoles of ONP produced per minute (Kroos L. et al.) according to the equation:

$$\frac{213 \times A_{420}}{[ml][mg \text{ protein/ml}][min]}$$

where 213 is derived from the extinction coefficient of ONP and converts  $A_{420}$  into nanomoles. [ml] and [mg protein/ml] refer to the sample of sonicate, [min] is the time the assay was incubated before stopping the reaction.

## 2-15 DNA extraction from M. xanthus

### 2-15-1 Preparation of chromosomal DNA

Chromosomal DNA was isolated by running a caesium chloride gradient of a total lysate of M. xanthus cells.

A dense culture of cells ( $A_{600}=1$ ) 10ml in volume was spun down in a universal at 5000rpm in a benchtop centrifuge. The supernatant was poured off and the pellet resuspended in 1ml of STE (25% sucrose, 50mM Tris.HCl, 5mM EDTA pH8.0). To this was added and mixed by inversion: 5 $\mu$ l of 20mg/ml proteinase K, 200 $\mu$ l of 0.5M EDTA and 125 $\mu$ l of 10% N-lauroylsarcosine. The tube was incubated at 50°C overnight. The volume of lysate was increased to 5.5ml with TE (10mM Tris.HCl pH8, 1mM EDTA). Into this was dissolved 5.7g CsCl and 0.55ml of 10mg/ml ethidium bromide was added. A 5.5ml Quickseal ultracentrifuge tube was filled completely with this solution then sealed and spun for 16 hours at 55000rpm in a VTi65.2 rotor (Beckman) in an ultracentrifuge.

The band of chromosomal DNA was removed using a syringe and needle. The ethidium bromide was removed by repeated extraction with isopropanol which had been equilibrated with water saturated with NaCl. The CsCl was then removed by dialysing against 2-3 changes of TE for at least 6 hours. The resulting DNA solution could be concentrated, if necessary, by air drying the dialysis bags.

2-15-2 Preparation of closed circular DNA from H. xanthus

The DNA circles released by homologous recombination were isolated as supercoiled circles in the same manner as plasmid was extracted from E. coli. A stationary phase culture of 500ml volume was spun down at 7500rpm in an MSE 6x250 rotor. The pellet was washed in 20ml of TES (50mM NaCl, 50mM Tris.HCl pH8, 5mM EDTA). The pellet was resuspended in 10ml of STE and transferred to a 40ml Oakridge tube. To the suspension was added 2.5ml of 0.5M EDTA and the mixture left on ice for 10 minutes. Ice cold Triton lysis mix (0.1% Triton X-100, 50mM Tris.HCl, 50mM EDTA pH8.5) was then added to lyse the cells. The tube was then spun at 19000rpm for 35 minutes in an MSE rotor in an MSE "Highspin" centrifuge. The pellet was removed using a hooked pasteur and 28.5g of CsCl dissolved in the cleared lysate. TE was added to increase the volume to 35ml then 1ml of 5mg/ml ethidium bromide was added. The mixture was kept on ice for an hour and the resulting precipitate pelleted by centrifugation at 15000rpm at 4°C for 15 minutes. The supernatant was transferred to a 40ml Quickseal tube by pouring through a syringe and needle with a layer of glass wool in the syringe to filter out any solids. The tube was spun for 16 hours at 45000rpm in a VT150 rotor (Beckman). A band of chromosomal DNA was apparent on the gradient. The part of the gradient below the band, presumed to contain supercoiled circular DNA, was allowed to run off through a syringe needle into an Oakridge tube, but carefully avoiding the chromosomal DNA. The sample was diluted in twice its

volume of TE, glycogen carrier added and the DNA precipitated by adding ethanol. The pellet was then dissolved in TE and used to transform E. coli.

#### 2-16 Isolation of plasmid from E. coli

##### 2-16-1 Rapid small scale isolation of plasmid DNA

A simplified version of the boiling method (Maniatis T. et al. 1982) was used.

Cells were grown overnight as 2cm<sup>2</sup> patches on LB plates. Each patch was scraped off using a toothpick dipped in TES into 0.33ml STET (8% sucrose, 0.5% Triton X-100, 50mM EDTA, 50mM Tris.HCl pH8) in an Eppendorf tube. To this was added 10µl of 0.33mg/ml lysozyme and the mixture vortexed for 3 seconds. The tube was transferred for 3 minutes to a boiling water bath that had just gone off the boil. The tubes were then spun in a microfuge for 15 minutes. The pellet was then removed using a hooked pasteur. The volume of supernatant was increased to at least 0.2ml with TE then 0.33ml of isopropanol added and mixed by vortexing. The tube was kept at -20°C for 2 hours then spun in a microfuge for 15 minutes. The supernatant was removed as completely as possible and the pellet dried in a vacuum. The pellet was dissolved in 50µl of TE and could be stored at -70°C. For restriction analysis, 4-6µl of the preparation was used per digest.

## 2-16-2 Large scale preparation of plasmid DNA

Larger quantities of plasmid were prepared by purification on a caesium chloride gradient (based on Maniatis T. et al. 1982). The method used was similar to that described in 2-15-2 except that 1ml of 10mg/ml lysozyme in 0.25M Tris.HCl pH8 was added and the tube kept on ice for 10 minutes before the addition of the lysis mix.

In this instance, the band of supercoiled DNA (here consisting of plasmid) was clearly visible; either as the lower of two bands or as the only band.

## 2-17 Transformation of E. coli

Cells were transformed by plasmid DNA using a method based on that of Mandel M. and Higa H. (1970). A 1ml sample of overnight culture of cells was diluted in 100-200ml SOB medium in a 2 litre flask and incubated at 37°C with vigorous shaking. When the cells had reached a density of  $A_{550}=0.2$  (for recA<sup>+</sup> strains and 0.4 for recA<sup>-</sup> strains) the culture was chilled on ice for 10 minutes. The culture was then divided between plastic (and therefore detergent free) universals which had been chilled. These were spun in an MSE Chillspin at 3000rpm for 10 minutes. The cells were resuspended in a half the original volume of chilled 0.1M CaCl<sub>2</sub>. This suspension was kept chilled for 15 minutes and then centrifuged as before and resuspended in 1/15 the original volume of 0.1M CaCl<sub>2</sub>. This mixture was aliquotted (0.2ml) into prechilled 1.5ml Eppendorf



tubes. To these was added DNA for transformation, not more than 40ng/aliquot. The DNA was always added in a large volume (at least 50 $\mu$ l of TE) to ensure adequate mixing. The tubes were kept on ice for 30 minutes then heat shocked at 42°C for 2 minutes. To each tube was added 1ml of SOB broth and the tube incubated without shaking at 37°C for 90 minutes. The contents were then spread onto well dried LB plates containing the appropriate antibiotic and the liquid allowed to absorb. Transformant colonies were obtained after overnight incubation.

#### 2-18 Restriction endonuclease digestion

Restriction digests were carried out under the conditions recommended by the suppliers. In addition to the recommended buffers, 1mM dithiothreitol (DTT) and 0.5mM spermidine were also added to the reaction mixtures.

#### 2-19 Alkaline phosphatase

Calf intestine alkaline phosphatase was used to dephosphorylate the 5' staggered ends of restriction enzyme cleaved DNA molecules. To a completed restriction digest was added 1/10th volume of 0.5M glycine/NaOH pH9.4 followed by 1/10 volume 10mM MgCl<sub>2</sub>, 1mM ZnCl<sub>2</sub>. Finally, 1 $\mu$ l of alkaline phosphatase was added to the mixture and it was incubated at 37°C for 30 minutes. E. coli alkaline phosphatase was used for dephosphorylating blunt ended fragments.

The same conditions were used except that the temperature was 65°C.

#### 6-20 Use of Exonuclease III to create deletion mutants of plasmids

This procedure was used to inactivate the kanamycin resistance gene of the promoter probe plasmid prior to Tn5 mutagenesis. The method is illustrated in Fig. 6-2. The plasmid was digested with HindIII then ethanol precipitated. The pellet was resuspended in 0.1ml ExoIII buffer (50mM Tris.Cl pH8.0, 5mM MgCl<sub>2</sub>, 10mM β-mercaptoethanol). To this was added 750 units of Exonuclease III (Boehringer) and the sample incubated at 37°C. Aliquots of 5μl were taken from the reaction at varying intervals: 30s to 30 minutes from the start of the reaction. The aliquots were immediately added to 15μl of 0.2M NaCl, 5mM EDTA to stop the reaction. After all samples had been taken, they were heated to 70°C for 10 minutes, then ethanol precipitated. The pellet from ethanol precipitation was resuspended in 50μl of S1 buffer (30mM sodium acetate, 50mM NaCl, 1mM ZnCl<sub>2</sub>. Then, 50 units of S1 nuclease were added. The reaction was incubated at room temperature for 30 minutes before being stopped by adding 6μl of 500mM Tris.Cl pH8.0, 125mM EDTA. The mixture was then phenol extracted and ethanol precipitated. The S1 nuclease removes the single stranded region created by the Exonuclease III but leaves the DNA with staggered ends. To create blunt ended DNA, the pellet was resuspended in 11μl of TE. To this was added 1.5μl of 10xTA buffer (330mM Tris acetic acid pH7.9, 660mM KCl, 100mM Magnesium acetate) and 4 units of T4 DNA

polymerase added. The 4 nucleoside triphosphates: dATP, dGTP, dCTP and TTP, were added (1.5µl of a 0.125mM solution of all four). The mixture was incubated at room temperature for 20 minutes, then precipitated using ammonium acetate (5 minutes at room temperature) <sup>+ Ethanol</sup>. Such a procedure precipitates DNA and leaves the unincorporated nucleotides in solution. The pellet was redissolved and the linear DNA ligated to itself (in a 20µl volume).

#### 2-21 Ligations

The enzyme used was T4 DNA ligase (Amersham) and the buffer, that recommended by the suppliers. For ligations of cohesive ends, 0.2µl of enzyme were used in a reaction. For blunt ended ligations, 0.5µl was used. Reactions were always carried out at 16°C overnight. The concentrations of insert and vector DNA were those recommended by Legerski R.J. and Robberson D.L. (1985). The reaction volume was 5µl for 1st order ligations (where two molecules were required to combine) and 20µl for zero order ligations (involving recircularisation of a molecule).

#### 2-22 Labelling of DNA by nick-translation

The method was based on that of Maniatis T. et al. (1982). Less DNA was used, 200ng as a rule. DNaseI was added to the reaction (1ng/µl). The reaction was incubated at 16°C for 3 hours, then the reaction was stopped by adding 2µl of 0.5mM

EDTA. Denatured salmon sperm DNA was added as a carrier and the unincorporated radionucleotide removed on a spun column containing Sephadex G25 in TE (Fig. 2-1). The labelled DNA could be stored frozen until ready for use.

#### 2-23 Phenol extraction

Protein was removed from DNA using Darbyshire Reagent. This is a solution of 100g phenol, 100ml chloroform, 4ml isoamyl alcohol, 0.1g 8-hydroxyquinoline. It was equilibrated with 2 x 40ml changes of Tris.HCl pH8 followed by 2 changes of TE. Stored under TE at 4°C, it was allowed to reach room temperature before use.

Phenol extraction was typically used to remove restriction or other DNA modifying enzymes before proceeding to the next stage of a reaction. The volume of the solution was increased to at least 0.1ml and 1µl of 0.5M EDTA added. It was then vortexed with an equal volume of Darbyshire Reagent. The upper aqueous layer was removed and extracted 4 times with diethyl ether to remove phenol and chloroform. The DNA was then precipitated with ethanol.

#### 2-24 Ethanol precipitation

Ethanol precipitation of DNA was carried out according to Maniatis *et al.* (1982). Salts were added: either sodium acetate to a final concentration of 0.1M or ammonium acetate to a concentration of 1M. Two volumes of ethanol were then

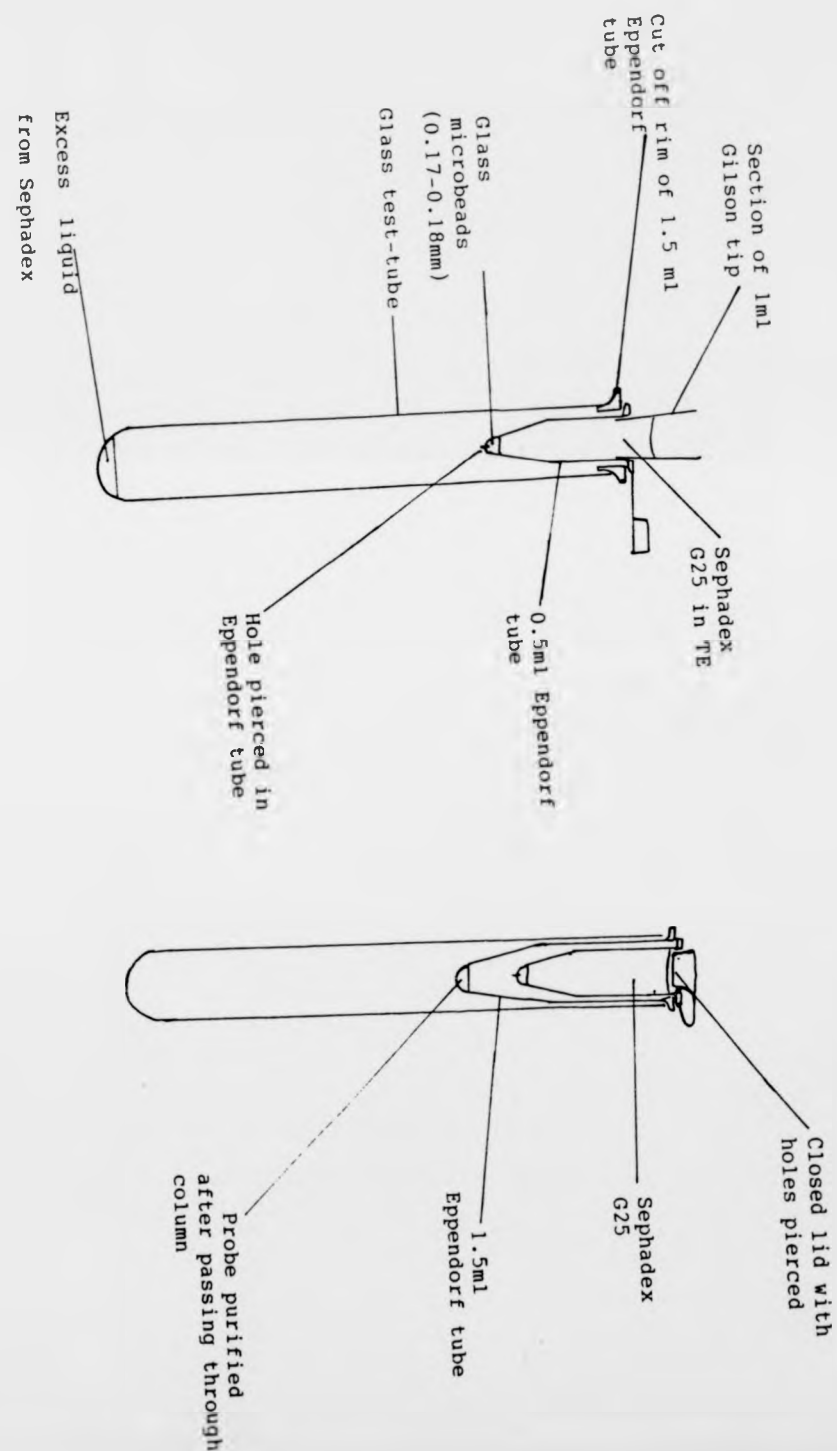
Fig. 2-1

Spun column technique used to purify DNA labelled by nick-translation.

a.) shows the method used to prepare a G25 spun column. The assembly is spun in a benchtop centrifuge with a swinging bucket rotor at 2500rpm.

b.) shows the arrangement for removing unincorporated nucleotides from the labelled probe. The 0.5ml Eppendorf tube prepared in a.) now contains compacted Sephadex. This tube is placed as shown in an intact 1.5ml Eppendorf tube and the completed nick-translation reaction pipetted onto the column. The cap is closed and the assembly spun at 2500 rpm for 10 minutes.

Fig 2-1



added. The temperature and time allowed for precipitation was dependent upon the DNA concentration (Crouse J. and Amorese D. 1987). Where the quantity of DNA was low (<2 $\mu$ g), 10 $\mu$ g of phenol extracted glycogen was added as a carrier (Hodgson D.A. unpublished method).

#### 2-25 agarose gel electrophoresis

Flat bed agarose gels were run as described by Maniatis T. et al. (1982). The buffer used was TBE (0.089M Tris, 0.089M boric acid, 2.5mM EDTA). All gels were run with ethidium bromide in the buffer (0.5 $\mu$ g/ml).

#### 2-26 Elution of DNA from agarose gels

Bands containing DNA were cut out of gels and eluted into 7.5M ammonium acetate using an IBI electroeluter (International Biotechnologies Inc.) used according to manufacturers' instructions.

#### 2-27 Southern transfer

DNA in gels was transferred to nitrocellulose by Southern transfer. The method was that described by Maniatis T. et al. (1982). Nitrocellulose paper was obtained from Amersham.

## 2-28 Colony transfer

M. xanthus colonies were lysed in situ and the DNA transferred to nitrocellulose using a technique devised for E. coli (Grunstein M. and Hogness D. 1975). However, it can be simplified for M. xanthus as no lysozyme treatment is necessary (O'Connor K. and Zusman D.R. 1983).

Colonies were picked onto DCY plates, 50 per plate. Cells were allowed to grow for 1-3 days. A circular piece of dry nitrocellulose paper cut to fit over the agar surface was placed over the colonies and left for several hours. The nitrocellulose was then carefully removed and immersed in 2 changes of 0.5M NaOH, for 2.5 minutes each. The nitrocellulose was then immersed in 2 changes of 0.5M Tris pH8 for 2.5 minutes each. The nitrocellulose was then given 2 changes of 2 x SSC (20 x SSC is 3M NaCl, 0.3M sodium citrate), followed by 2 changes of 95% ethanol. The nitrocellulose was then air dried. There was no need to oven dry the nitrocellulose in this case.

## 2-29 Hybridisation conditions

Nitrocellulose filters, whether from Southern transfer or from colony transfer, were hybridised with radioactive probe labelled by nick translation. The method was based on that of Maniatis T. et al. (1982) for hybridisation in the absence of formamide. The solution used both for hybridising and

prehybridising was 5 x SSC, 0.02M Tris.Cl pH7.4, 0.5% SDS and 0.1mg/ml denatured herring sperm DNA. The nitrocellulose was prehybridised for 1 hour in 10ml of this solution at 67°C. This prehybridisation step was not necessary when hybridising the colony transfers. The probe, labelled by nick translation, was denatured by adding 1/10 volume of 10N NaOH and then neutralised by adding 1 volume of 2M Tris.Cl pH6.8. The probe was then added to 20ml of hybridisation solution. The nitrocellulose was hybridised overnight at 67°C. The following day, the nitrocellulose was washed in 2 changes of 2 x SSC, 0.5% SDS at 67°C for 30 minutes each followed by 2 changes of 0.2 x SSC, 0.2% SDS at 50°C for 30 minutes each.

The nitrocellulose was air dried and sealed in a thin polythene bag. It was autoradiographed with X-ray film together with an intensifying screen at -70°C.



### CHAPTER 3

Preparation and  
identification of  
developmentally regulated  
gene fusions

### 3-1 Introduction

Gene fusions were prepared by constructing a library of cloned chromosomal DNA from M. xanthus. The library was constructed in a promoter-probe plasmid which could be packaged in phage P1 and transduced to M. xanthus. In order to find developmentally regulated gene fusions, transductants were screened for  $\beta$ -galactosidase expression under conditions permitting vegetative growth and also under starvation conditions which induce sporulation. Several of the gene fusions were tested further and the gene fusions cloned.

### 3-2 Cloning of M. xanthus chromosomal DNA

The principle of the integrative promoter-probe plasmid was described in the Introduction (sections 1-3-4 to 1-3-6). The promoter-probe plasmid, used for cloning, was pDAH122 (Fig. 3-1). This contains genes conferring kanamycin and ampicillin resistance, a P1 inc region and the E. coli lac operon minus its promoter. Sequencing work performed in this lab. confirms that the lacZ promoter has been deleted but the translation initiation site is unaffected (McGowan S. unpublished)(Fig. 3-2). Upstream of this are translation termination codons in all three reading frames. Any gene fusions will therefore be with the cloned insert DNA and will be transcriptional rather than translational fusions. The trpA region from E. coli is a cloning artifact (Casadaban M.J. and

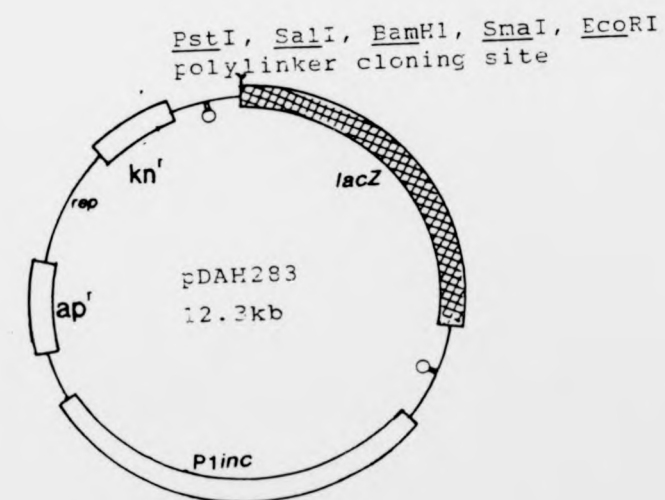
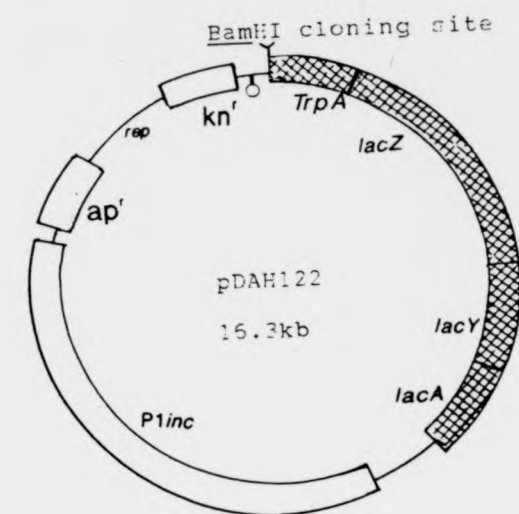
Fig. 3-1

Plasmid maps pDAH122 and pDAH283

For clarity, restriction sites are not shown except for those in the cloning sites of the plasmids.

A detailed restriction map of pDAH122 is shown in Fig. 5-5

The transcriptional reading frame of the *lac* operon is shown cross-hatched. The *lacZ* gene shown includes part of the *trpA* gene which is a cloning artefact. The positions of transcription terminators are indicated by stalked circles.



*lacZ* 5' Region

-35                      -30                      operator

ACCACG GCTTACACTT ATGCTTCGACTG TATGTT GTGTT    TGAATTTTACGCAATACAAATT T CACAC AGGA AACAGCT ATG ACC ATG ATT

RBS                      Met Thr Met Ile

*trpA* 3' Region    Ala Ala Thr Arg Ser End

GCG GCG GCG ACG CGC AGT TAA TCCACACGCGCCGCAAGTTCCGCTGCGCGGCAATTTTAACTTCTTTA ATGAGCCGCAAAATCTTAA

*trpA/lacZ*                      Ala Ala Thr Arg Ser End

Fusion ΔW205    GCG GCG ACG CGC AGT TAA TCCACACGCGCCGCAAGTTCCGCTGCGCGGCAATTTTAACTTCTTTAT CACAC AGGA AACAGCT ATG ACC ATG ATT

RBS                      Met Thr Met Ile

Sequence of the *trpA/lacZ* fusion point of ΔW205 of Casadaban *et al* 1980

RBS=ribosome binding site

Fig. 3-2

Sequence of the *trp-lac* region (McGowan S. and Hodgson D.A., unpublished)

The sequence data shown here indicate that the *lacZ* promoter has been deleted without affecting the ribosome binding site and translation initiation codon of the *lacZ* gene.

Cohen S.N. 1980). Further upstream is a unique BamHI site for cloning. The 5' staggered ends produced by cleavage with BamHI are identical to those produced by Sau3AI. Upstream of the cloning site is a transcription terminator from E. coli, known to function heterologously in M. xanthus (Hodgson D.A. unpublished).

Chromosomal DNA was prepared from a 500ml stationary phase ( $A_{600} > 1.0$ ) culture of strain DK101. A partial digest was performed on 80µg of DNA using 20 units of Sau3AI for 90 minutes. This was then size fractionated by agarose gel electrophoresis (Fig. 3-3). The region containing fragments between 3 and 6kb in length was cut out of the gel, electroeluted and precipitated with ethanol. This particular size fraction was chosen for cloning as it has a high probability of recombination when reintroduced into M. xanthus (O'Connor K.A. and Zusman D.R. 1986). The fragments were also of sufficient length so that the cloning of a fragment internal to an operon would be unlikely. Such a fragment, if transduced into M. xanthus, would result in gene inactivation (Fig. 1-4).

The plasmid was digested with an excess of BamHI, and the resulting 5' ends dephosphorylated to reduce the chance of plasmid self ligation. This was ligated to the Myxococcus chromosomal fragments. Kanamycin resistant recombinant clones were obtained by transformation into E. coli strain MC1061. A control ligation, using plasmid only, showed that there was only a minimal background of self ligated plasmid (Table 3-1).

Plasmid DNA was prepared on a small sample of the colonies from the recombinant plates. This was digested using EcoRV

TABLE 3-1

Transformants obtained after ligating chromosomal *M. xanthus* DNA to the vector pDAH122

Each plate was prepared with *E. coli* strain MC1061 transformed with an aliquot of the ligation reaction containing 40ng of DNA.

plate	number of colonies
1	790
2	517
3	624
4	1232
5	582
6	718
	<hr/>
Total	4463

A control ligation consisting of vector without the insert was also transformed. When 40ng of this were used in transformation 34 colonies were obtained.

TABLE 3-2

Expression of  $\beta$ -galactosidase by *M. xanthus* containing gene fusions through transduction with the promoter-probe library

Media on which expression occurs	number of colonies
DCY (vegetative) only	59
CF (sporulation) only	15
both media	35
neither medium	77
	<hr/>
total	186

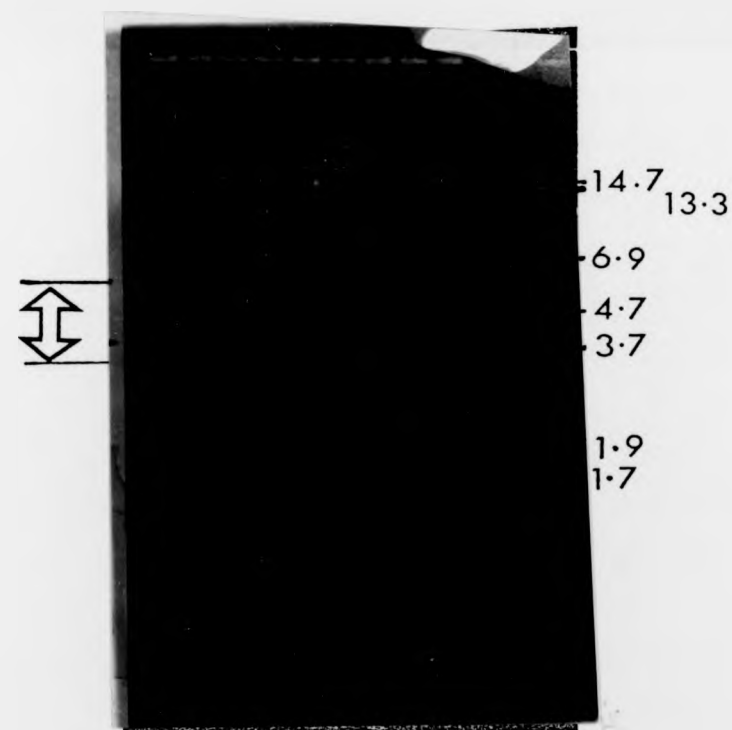
Fig. 3-3

Partial digestion of *M. xanthus* DNA for cloning in a promoter-probe plasmid

Chromosomal DNA from strain DK101 (80µg) was digested with 20 units of *Sau*3HI. Reaction time was 30 minutes. Previous experiments (not shown) had demonstrated that this ratio of enzyme to DNA, produced a good yield of fragments of the required size.

The reaction was stopped and the reaction mix divided between seven tracks of the gel. The remaining track contains an *Ava*I digest of  $\lambda$  DNA as a size marker. The sizes of the marker fragments are shown alongside the photograph. The vertical arrows show the region of the partial digest subsequently cut out of the gel and purified by electroelution.

Fig. 3-3



and BamH1 combined. It was apparent that a range of different fragments had been cloned.

3-3 Transductants in M. xanthus show differential expression of  $\beta$ -galactosidase

The colonies from each plate were suspended in a small volume of LB broth using a glass spreader, and an overnight culture prepared of all the colonies from each of the six plates. A sample of this plasmid library was stored in glycerol at  $-70^{\circ}\text{C}$ . From the remaining culture, a dilution was taken, packaged into P1 phage and used to transduce M. xanthus strain DK101 to kanamycin resistance. Many thousands of colonies were obtained.

Transductants were picked onto both DCY (vegetative) and CF (sporulation) medium. After 5 days, they were sprayed with 10mg/ml 4-MUG in DMSO and examined for blue fluorescence. There were considerable differences in expression between the two media (Table 3-2).

Although there is clearly a great difference between the patterns of gene expression on rich medium and on clonal fruiting medium, it is possible that many of these changes are unrelated to development and sporulation. A sudden reduction in nutrient levels is likely to lead to a stringent response, as in E. coli (Cashel M. and Rudd K.E. 1987). This results in the shutting down of expression of many genes but also leads to an increase in expression of others. Another source of confusion arises from the rapid growth of cells on complex medium. By the time sporulation had occurred, the control



colonies on rich medium had undergone many rounds of cell division. The differences in cell numbers would render unreliable any visual screen for enzyme activity.

To overcome these difficulties, A1 (minimal) medium was used. The availability of nutrients is only just enough to support vegetative growth without sporulation taking place. Indeed, it was observed that strain DK1622 formed raised mounds, reminiscent of nascent fruiting bodies, when transferred from DCY medium to A1 medium but these soon dispersed to leave a typical vegetative cell swarm. Cell growth was much slower on A1 medium than on DCY. Doubling time is 22-36 hours on A1 medium compared with 3-5 hours on complex media such as DCY (Rosenberg E. 1984).

A much larger number of transductants were then screened by picking them onto A1 medium and CF medium. After 4-5 days they were sprayed with 2mg/ml 4-MUG in DMSO and examined for fluorescence and whether there was any increase in activity when under sporulation conditions. The results of this screening are shown in Table 3-3 in the column "Transduction 1".

The transductants which showed increased expression during sporulation were retested to confirm the results. This was done by spraying plates with 4-MUG as before and also by using plates containing X-gal in the medium and observing the blue colour released by  $\beta$ -galactosidase activity. Many of the original observations of sporulation regulated expression were not reproducible. However, there were 5 transductant strains which showed interesting profiles of expression. Quantitative assays of the enzyme were performed on these. Cell suspensions were spotted onto CF plates and allowed to sporulate. In

Table 3-3

	1	Transduction 2	3	4
Maximum number of gene fusions in phage lysate	4463	1307	537	372
Total $Kn^r$ transductants tested	1150	300	1657	>1000
Transductants expressing $\beta$ -galactosidase during growth	632	N.R.	N.R.	N.R.
Expression increased during development	49	9	111	99

\* = screened by D.H. Hodgson

N.R. = not recorded

Numbers of transductants in *M. xanthus* obtained by transduction with promoter probe libraries. Transduction 1 used phage prepared from the library described in section 3-1. Transduction 2 used phage prepared from a portion of the same library and is described in 3-5. Transductions 3 and 4 were prepared from libraries prepared by D.H. Hodgson. The library used in 3 was made using chromosomal fragments greater than 7kb in length. Transduction 4 was made using fragments between 4 and 7kb in length.

## TRANSDUCTION 1.

Strain	UWH1	UWH2	UWH3	UWH4	UWH5
Vegetative expression	298.2	213	340.8	42.6	149.1
Sporulation expression	170.4	426	21.3	213	127.8
% Increase during sporulation	-43	100	-94	500	-14

## TRANSDUCTION 3.

Strain	IV-3-2
Vegetative expression	109.2
Minimal medium	184.2
Sporulation expression	115.0
% Increase during sporulation	5.3

## TRANSDUCTION 2.

Strain	A45	D32	A48	E8
Vegetative expression	347.1	427.6	101.2	74.4
Sporulation expression	1831.8	3280.2	136.32	138.4
% Increase during sporulation	420	670	35	86

## TRANSDUCTION 4.

Strain	V-1-3	V-24	V-1-20
Vegetative expression	1467.6	100.3	253.5
Minimal medium	3940.1	176.8	843.5
Sporulation expression	2279.1	225.8	575.1
% Increase during sporulation	55	125	126

Table 3-4

Activity of  $\beta$ -galactosidase/mg soluble protein of transductant strains isolated in a screen for increased expression during development as summarized in Table 3. The units used are nmoles ONP released/mg of soluble protein. Vegetative expression is the activity of cells from a broth culture measured before spotting onto sporulation medium. Sporulation expression is the maximum expression measured during a sampling timecourse on sporulation medium (Lf or 1M). The increase in expression encountered during sporulation is also shown. The activity obtained on M1 (minimal) medium is shown in the cases where this was measured.

addition, cell cultures were induced to sporulate by the glycerol method. In both cases, samples were taken at intervals and these were sonicated and then assayed for both  $\beta$ -galactosidase and protein (Figs. 3-4-1 to 3-8-3 and Table 3-4). The units of specific activity are those described in Materials and Methods (2-14). Some of the graphs also show results from strains constructed by transducing the gene fusion to another strain (DK1622 or DK101). These results are discussed in section 3-5-2.

Strain UWM1 showed a decay in activity both during starvation induced sporulation (Fig. 3-4-1) and during glycerol induced sporulation (Fig. 3-4-2). This gene would appear to be switched off during sporulation. Strain UWM2 showed an increase in expression early in development (Fig. 3-5-1). However, there was a different effect in this strain when sporulation was induced by glycerol (Fig. 3-5-2). After high levels of expression early during induction of sporulation, the level of expression later dropped. Strain UWM3 showed a small increase in expression when induced to sporulate with glycerol (3-6-2), but no increase when under starvation conditions (3-6-1). Strain UWM4 showed increased expression during the later stages of starvation induced sporulation (Fig. 3-7-1). Development and expression was also induced when sporulation was induced by glycerol (Fig. 3-7-2). A different pattern existed in UWM5. Although there was a spectacular increase after glycerol induction of sporulation (Figs. 3-8-2 and 3-8-3), there was no comparable response during starvation induced sporulation (Fig. 3-8-1).

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Fig. 3-4-1

Expression of gene fusion in strain UWM1 during starvation induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto T1 medium. The point at time=0 was obtained from a sample taken from the broth culture before plating.

Fig. 3-4-2

Expression of gene fusion in strain UWM1 during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following the addition of glycerol to a log-phase culture.

Fig. 3-4-1

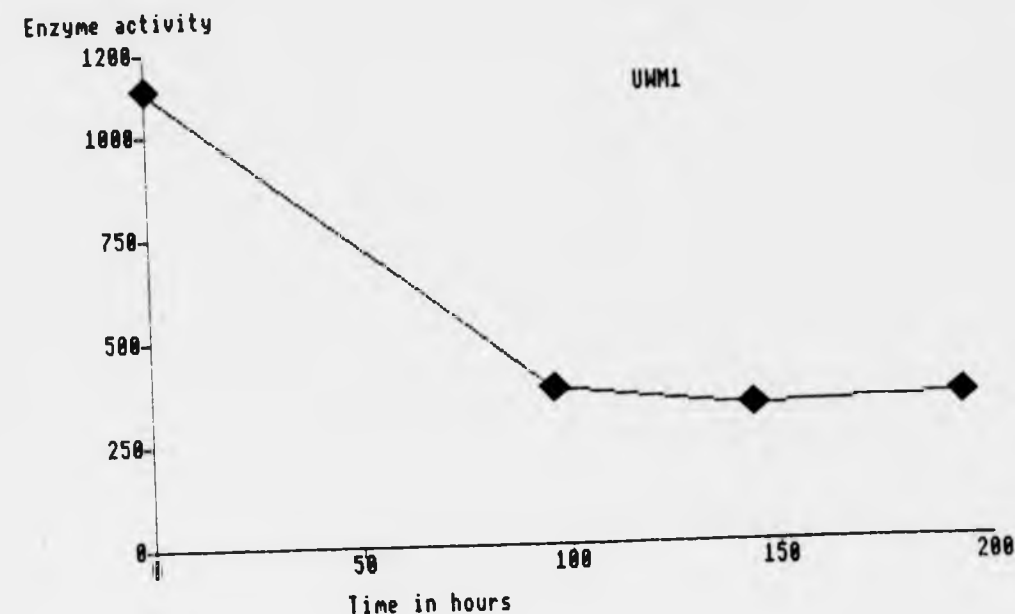


Fig. 3-4-2

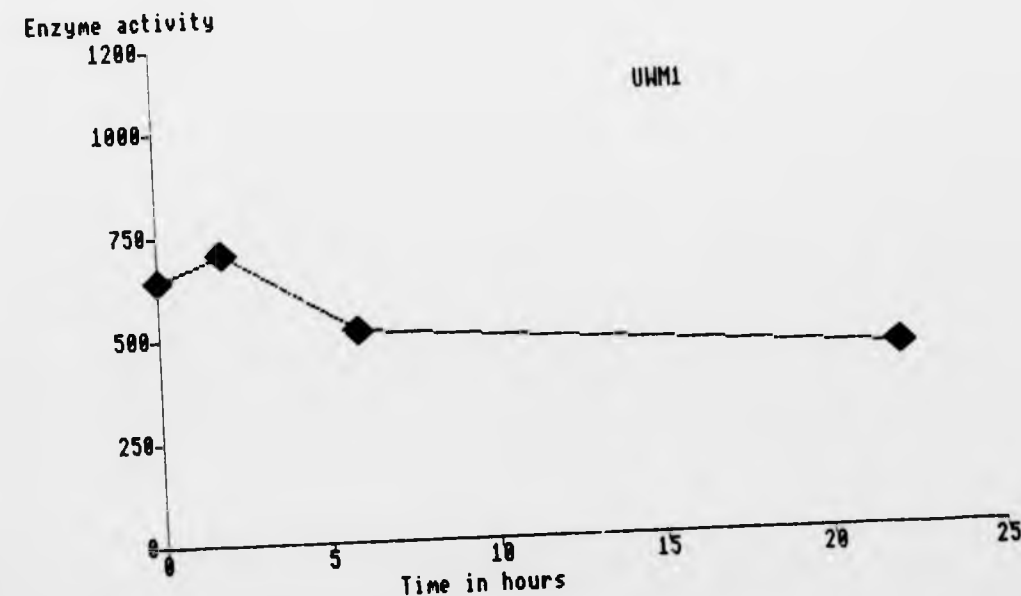


Fig. 3-5-1

Expression of gene fusion in strain UWM2 during starvation induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 was obtained from a sample taken from the broth culture before plating.

Fig. 3-5-2

Expression of gene fusion in strain UWM2 during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following the addition of glycerol to a log-phase culture.

Fig. 3-5-1

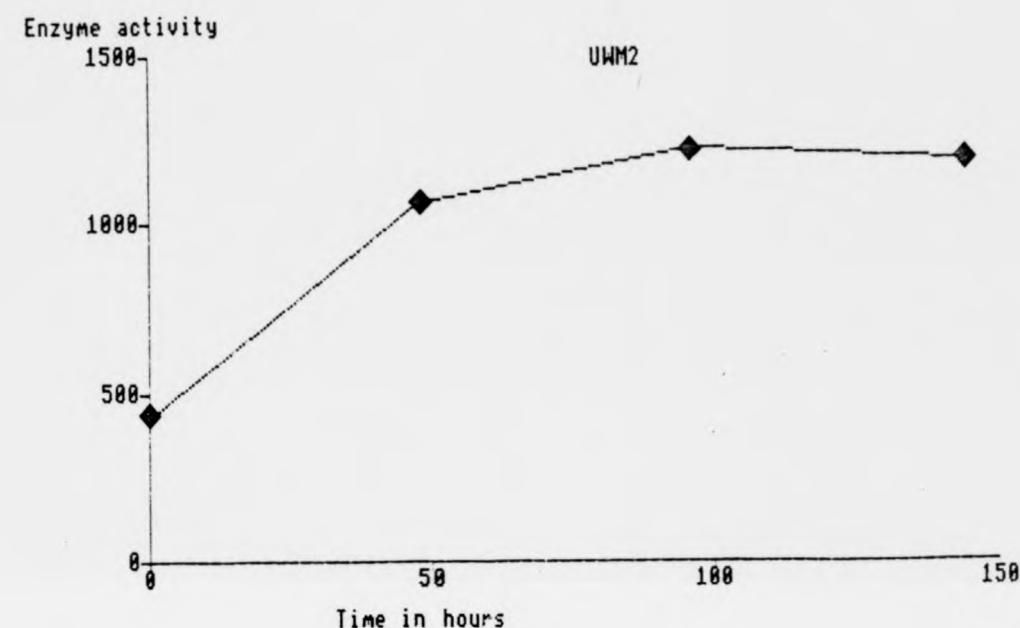


Fig. 3-5-2

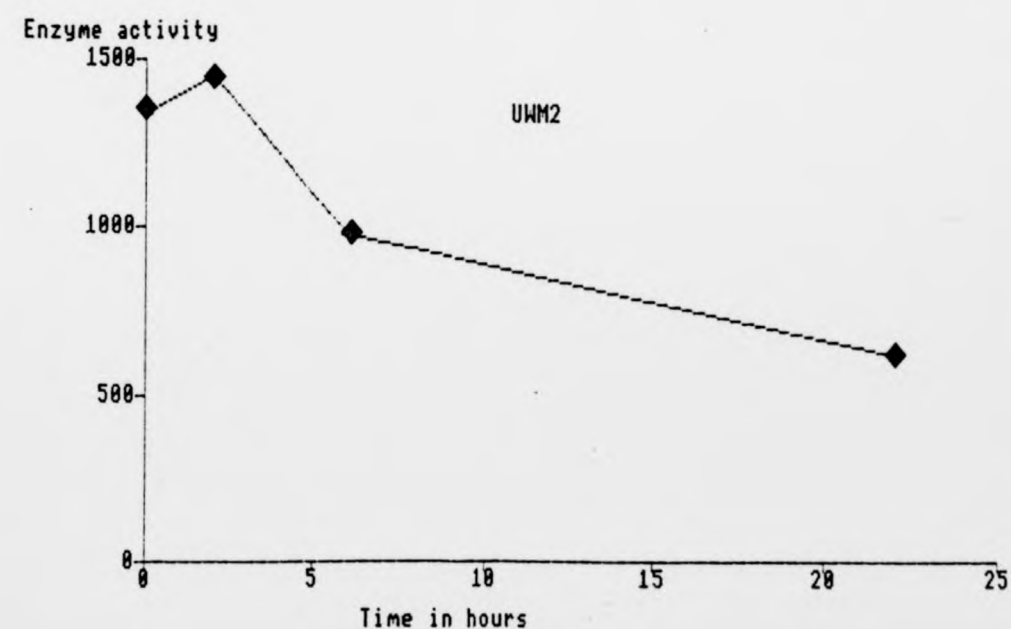


Fig. 3-6-1

Expression of gene fusion in strains UWM3 and DK1622[pUWM3] during starvation induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The points at time=0 were obtained from samples taken from broth cultures before plating.

Fig. 3-6-2

Expression of gene fusion in strains UWM3 and DK101[pUWM3] during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following the addition of glycerol to a log-phase culture.

Fig. 3-6-1

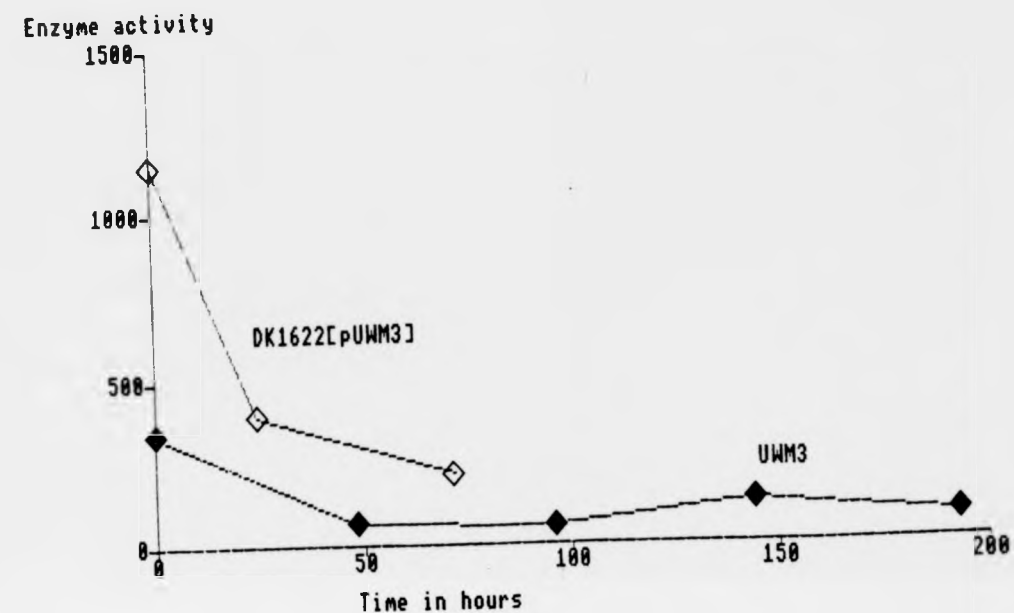


Fig. 3-6-2

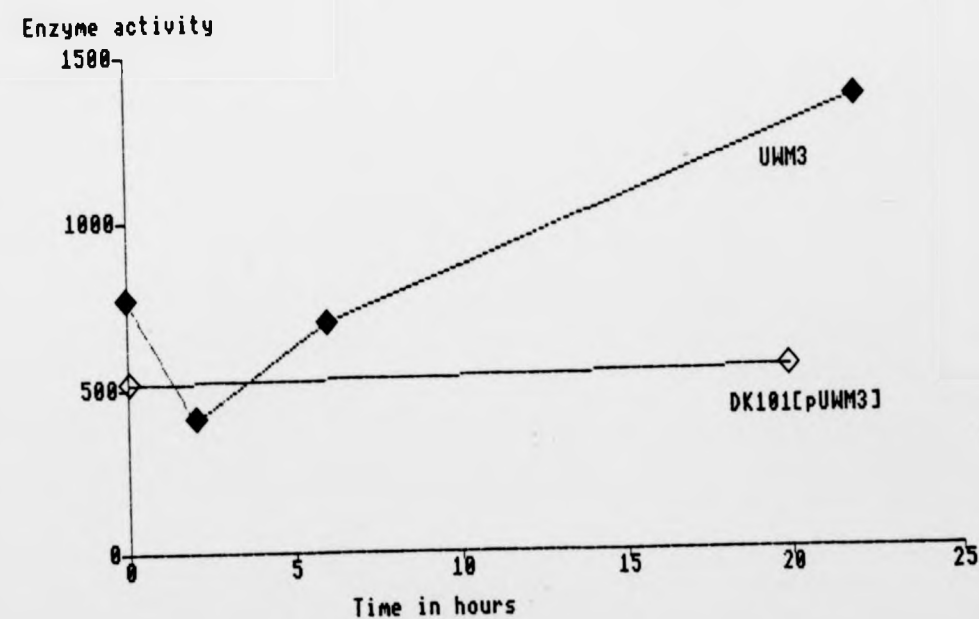




Fig. 3-7-1

Expression of gene fusion in strains UWM4 and DK1622[pUWM4] during starvation induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The points at time=0 were obtained from samples taken from broth cultures before plating.

Fig. 3-7-2

Expression of gene fusion in strain UWM4 during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following the addition of glycerol to log-phase culture.

Fig. 3-7-1

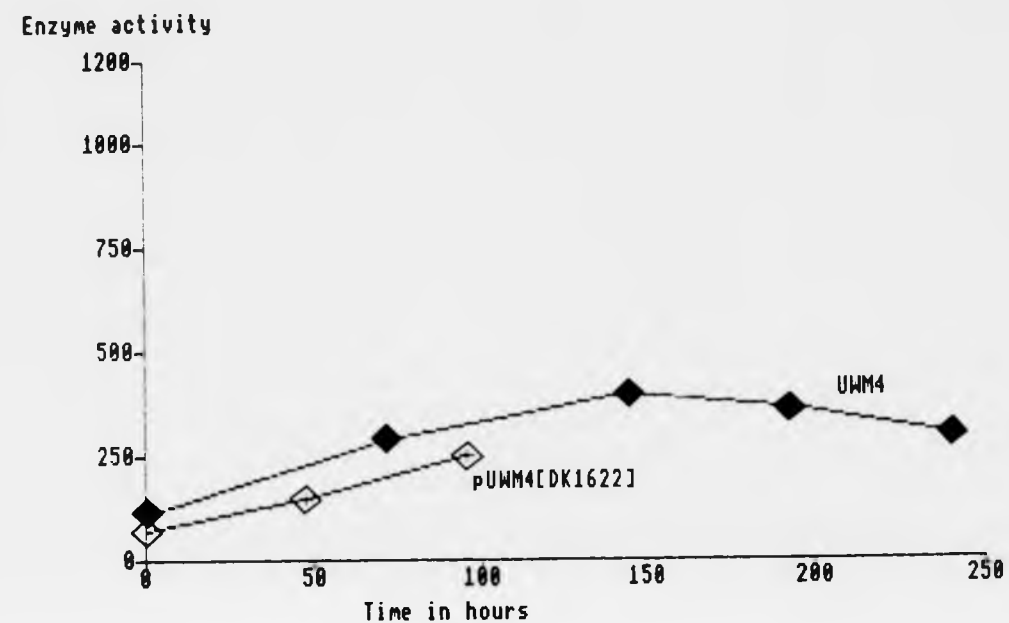


Fig. 3-7-2

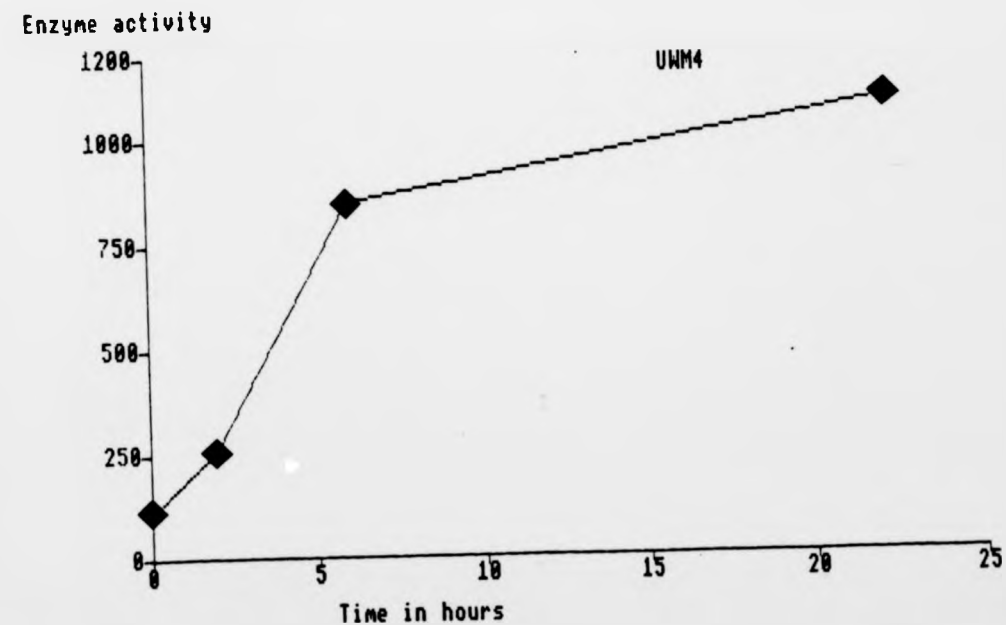


Fig. 3-8-1

Expression of gene fusion in strains UWMS and DK1622[pUWM5] during starvation induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 was obtained from samples taken from the broth culture before plating.

Fig. 3-8-2

Expression of gene fusion in strain UWMS during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14). In this case, the vertical axis is on a different scale to that in 3-7-1 in order to accommodate the very large observed increase in enzyme activity.

The horizontal axis shows the time in hours following the addition of glycerol to a log-phase culture.

Fig. 3-8-1

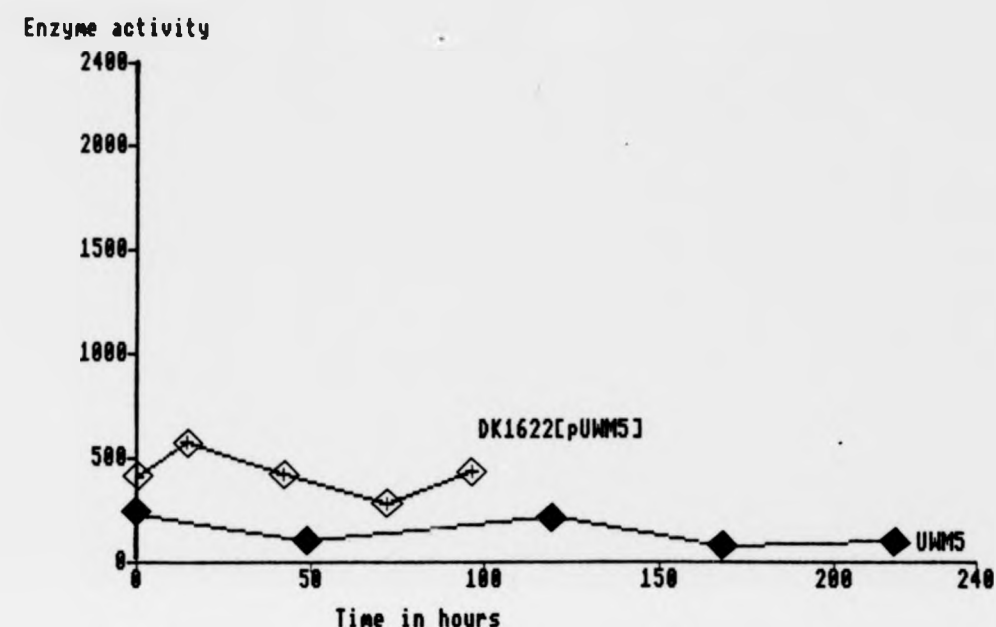


Fig. 3-8-2

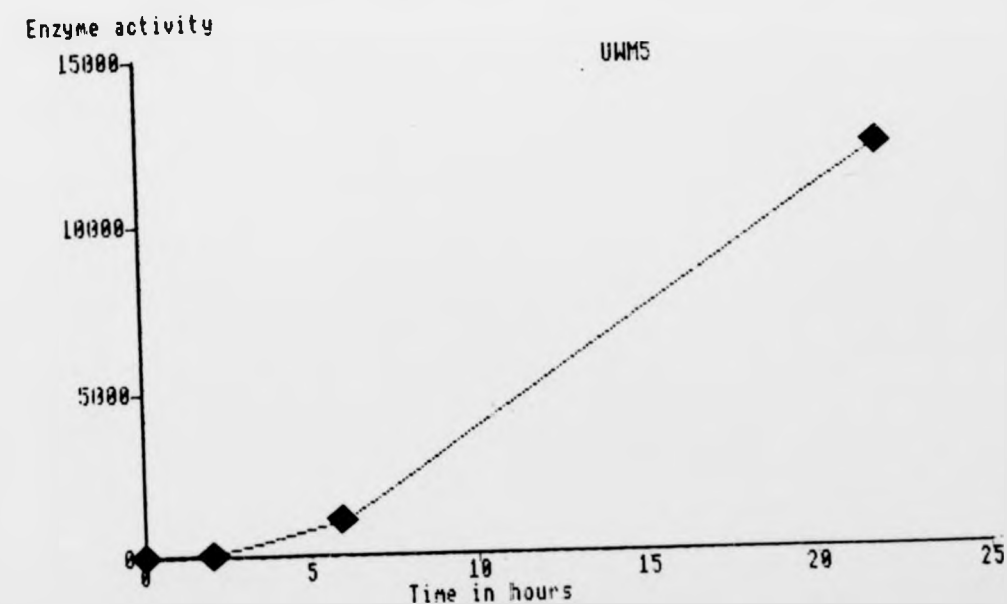
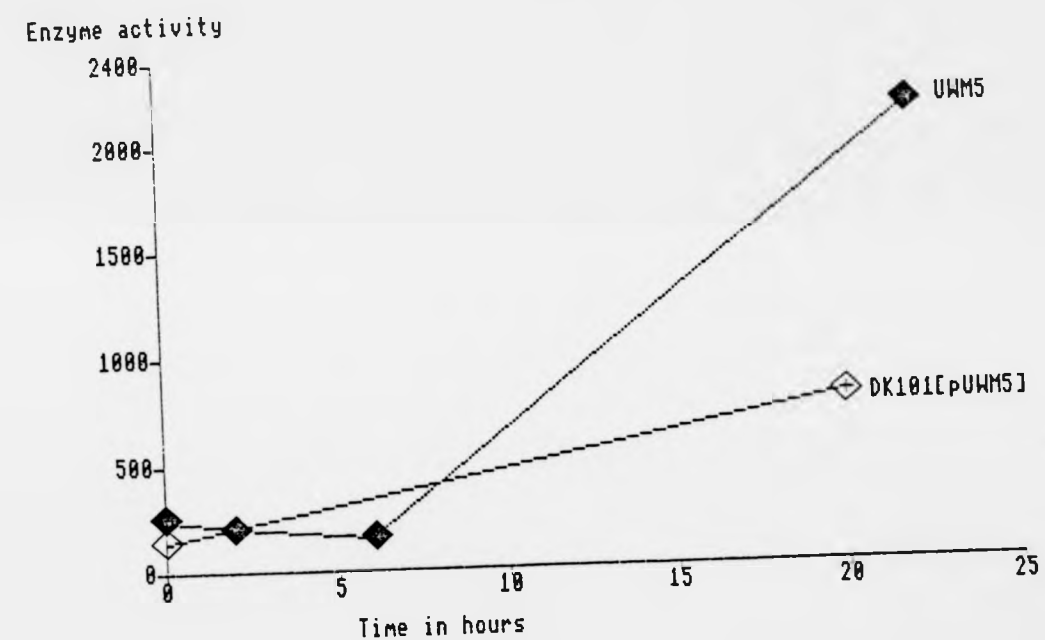


Fig. 3-8-3

Expression of gene fusion in strain UWM5 and DK101[pUWM5] during glycerol induced sporulation

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following the addition of glycerol to a log-phase culture.



3-4 Attempts were made to isolate additional gene fusions of interest

A second transduction experiment was carried out using the existing promoter probe library. The recipient strain was DK1622. This resembles a wild type strain in its motility and, in consequence, sporulates more rapidly and reproducibly than strain DK101. Transductants were screened as before on both A1 medium and CF medium. Transductants appearing to show increased expression during sporulation, were retested by growing suspension cultures in DCY medium, then resuspending the cells in TM and spotting them onto both A1 and CF medium containing X-Gal. The results are shown in Table 3-3 in the column headed "Transduction 2". Five transductant strains were assayed quantitatively for  $\beta$ -galactosidase expression (Table 3-4), and in two of these (A45 and D32) there was an increase in expression during development. However, the observed increases could not be reproduced in 2 subsequent repeat experiments.

A third selection of gene fusions was also screened. These were from a promoter probe library prepared by D.A. Hodgson using the vector pDAH283 (Fig. 3-1). This is similar to pDAH122 except that the size has been reduced by the removal of the lacY and lacA genes and part of the P<sub>lac</sub> region followed by adding back part of the P<sub>lac</sub> region. The cloning site has been augmented by the addition of a polylinker and has been brought closer to the transcription initiation site by the removal of most of the trpA region without affecting the translation initiation site of lacZ (Fig. 3-2).

The promoter probe library had been transduced into DK101 and transductants picked onto both A1 and CF medium. In this instance, transductants showing evidence of increased expression during development were picked and grown to a high density in DCY medium. A sample of each culture was kept for storage at  $-70^{\circ}\text{C}$  and another sample resuspended in TM and spotted onto A1 and CF plates containing X-gal. Some of these, indeed, showed increased expression during development (Table 3-3 columns 3 and 4). A selection of these were again spotted onto TM and A1 plates and assayed at intervals using ONPG (Table 3-4). Although small increases were observed by comparing cells from rich medium (CTTYE) with sporulating cells, the corresponding assays of cells from A1 (minimal) medium revealed that these low nutrient vegetative conditions also led to an increase in enzyme activity. The main objective is to find genes expressed during sporulation, rather than those induced under low nutrient conditions in the absence of sporulation. Consequently, these gene fusions were not studied further.

### 3-5 Cloning of gene fusions

3-5-1 Circular DNA formed spontaneously in M. xanthus can be cloned in E. coli

In the Introduction (1-3-6-2) it was explained how supercoiled circular DNA, corresponding to the original gene fusion, can excise from the chromosome and be isolated on a caesium chloride gradient. In this way, the promoter probe

containing the gene fusion can be cloned. Once this has been achieved, it is then possible to transduce it to other M. xanthus strains and observe its regulation under sporulation conditions.

Supercoiled circular DNA was isolated on CsCl gradients made from cleared lysates of the five M. xanthus strains UWM1 to UWM5 inclusive and also A45 and D32. The gradient fractions, assumed to contain supercoiled DNA, were ethanol precipitated and used to transform E. coli strain MC1061. Plasmid was prepared from several of the transformant colonies. Restriction analysis showed that they consisted of the original vector plus cloned DNA fused to the lacZ gene (Fig. 3-9).

3-5-2 The cloned gene fusions could be retransduced intact to M. xanthus

The plasmids isolated from strains UWM1, UWM2, UWM3, UWM4 and UWM5 and containing the cloned gene fusion are described by the initial p followed by the name of the original fusion strain. Hence pUWM4 is derived from UWM4. Strains UWM3, UWM4, and UWM5 had characteristic profiles of expression when under sporulation conditions. Although the gene fusions had been cloned, it was still not certain whether the clone contained all the sequences necessary for the observed regulation of gene expression. In order to investigate this, it was necessary to transduce the cloned plasmids back into M. xanthus. Plasmids pUWM3 and pUWM5 were packaged into P1 and transduced to M. xanthus strain DK101. In each case, the

Fig. 3-9

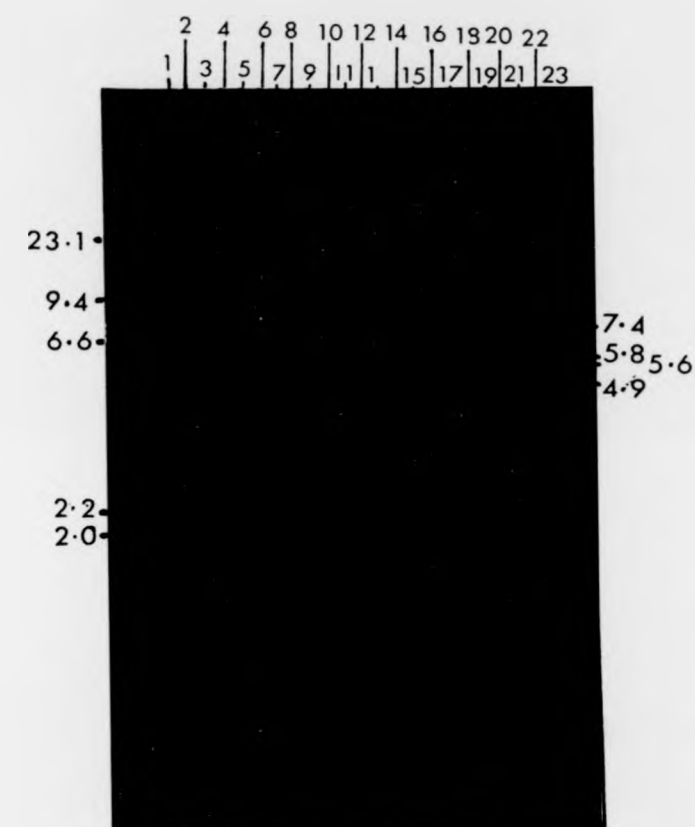
Restriction analysis of plasmids formed by spontaneous recombination within the *M. xanthus* chromosome

Plasmid DNA was cloned from strains PUWM1, PUWM3, PUWM4 and PUWM5 (see text 3-5-1). Plasmid DNA was prepared from several clones from each strain and digested with *EcoRV* and *XhoI* combined.

Two bands visible on every digest: 5.5kb and 7kb, are derived from the cloning vector pDHH122. Other fragments are variable in size and arise from sites within the cloned *M. xanthus* DNA.

Tracks	
1	<i>Hind</i> III digest of $\lambda$ DNA
2	<i>Hind</i> III/ <i>Eco</i> RI double digest of $\lambda$ DNA
3, 4, 5, 6 and 7	Clones derived from UWM1
8, 9, 10, 11 and 12	Clones derived from UWM5
13, 14, 15, 16 and 17	Clones derived from UWM4
18, 19, 20, 21 and 22	Clones derived from UWM3
23	<i>Eco</i> RI digest of $\lambda$ DNA

Fig. 3-9



pattern of gene expression during glycerol induced sporulation was investigated (Figs. 3-6-2 and 3-8-3). The plasmids pUWM4 and pUWM5 were transduced into wild type strain DK1622. This strain undergoes development more rapidly and reproducibly than DK101 and is therefore likely to allow a more realistic measure of developmentally regulated expression. Expression was measured during starvation induced sporulation (Figs. 3-6-1, 3-7-1 and 3-8-1). These results show that the patterns of developmentally regulated expression can be regulated by sequences which have been cloned in the plasmid. This does not exclude the possibility that additional cis acting sequences, upstream of the cloned region, are required for correct regulation. This is because the plasmid always integrates in the same location.

Starvation induced regulation is apparent in the strain DK1622[pUWM4] (Fig. 3-7-1). Glycerol induced regulation is apparent in strains containing pUWM5. (Figs. 3-8-2 and 3-8-3). There was little apparent regulation in the strain DK101[pUWM3] (Fig. 3-6-2).

In many of these cases: UWM3; UWM4; and UWM5 there were considerable differences between the original strain and the reconstructed strain both before and after sporulation. It is possible that these differences arise from differences in the genetic background. However, they could equally well be the result of subtle variations in the experimental conditions. This is apparent from the comparison of two separate experiments where strain UWM5 is induced to sporulate using glycerol (Figs. 3-8-2 and 3-8-3).



3-6 The strain UWMS contains a mutant allele affecting aggregation.

It was observed, during the induction of development and sporulation of the fusion strains UWM1 to UWMS, that UWMS did not form normal fruiting bodies. However, the strain DK101[pUWMS] consisted of the gene fusion transduced to an unmutated background. This exhibited normal development for DK101 (Fig. 3-10). Furthermore, a  $\text{Kn}^s$  strain, arising presumably from recombinational loss of the integrated plasmid, was isolated by streaking UWMS in the absence of selection. This strain (UWM1000) retained the sporulation defective phenotype. The mutant must, therefore, be unlinked to the gene fusion. The mutant developmental phenotype consists of flat patches, apparently of spores, surrounded by areas consisting of vegetative cells. The patches of spores are far smaller than true fruiting bodies and are not raised above the agar surface. However, the shape of the vegetative colonies resembled DK101 not a totally nonmotile mutant, such as DK306. In other words, the strain had not lost all motility. Such a phenotype has been previously reported in both aggregation mutants (Morrison C.E. and Zusman D.R. 1981) and motility mutants that have lost only one of the 2 motility systems (Hodgkin J. and Kaiser D. 1979b).

Fig. 3-10

## Photographs of fruiting bodies

Strains UWM5 and DK101[pUWM5] Cells were spotted onto TM medium and incubated at 33°C for 5 days to allow development and sporulation to take place. The fruiting bodies were photographed under a Wild binocular microscope.

Photographs were taken under transmitted illumination. Magnification is 30x

DK 101  
(pUWM 5)

UWM 5



## 3-7 Concluding remarks

The promoter probe library may well include most of the genome. The relationship between the number of recombinant clones, insert size and the probability of a gene's occurring in a library can be expressed as:

$$N = \frac{\ln[1-P]}{\ln[1-f]}$$

where N is the number of recombinant clones.

P is the probability of any one gene occurring in the library.

f is the fractional proportion of the genome in a single recombinant.

As the inserts average 5kb, the library containing 4300 recombinants should contain 97% of the genome. However, if transcriptional fusions to lacZ are considered, the fusion must occur within each gene and in one of two orientations, in order to obtain a transcriptional gene fusion. The proportion of transcription units of 0.5Kb, of either orientation, which are included in the library, will be 24%. For 2.0kb transcription units in either orientation this figure will be 67%. Such calculations assume that every part of the genome has an equal chance of being cloned. In fact, many clones may not appear in a high copy number vector such as pDAH122 because their expression, as a result of their own promoter activity, is lethal to E. coli. For this reason, the calculated probabilities are likely to be overestimates.

A total of four gene fusions consistently increase their expression when under sporulation conditions. Two of these,

UWM4 and UWM5, show large increases in expression during glycerol induced sporulation. The two genes, of unknown function, are identifiable as developmentally regulated gene fusions. The gene whose promoter has been characterised in pUWM4 was named isg-1, and the gene whose promoter has been characterised in pUWM5 has been named isg-2. The corresponding gene fusions have been named isg-1>lacZ and isg-2>lacZ.

Another strain, UWM3, shows a smaller increase in expression during glycerol induced sporulation than UWM4 or UWM5. A fourth, UWM2, shows a small increase in expression but only during starvation induced sporulation. A surprising result was that the increase in expression during glycerol induced sporulation in UWM3 and UWM5 was not in evidence during starvation induced sporulation. This is in spite of the fact that the fusion containing strains were originally isolated as a result of a screen for starvation induced expression and that the increase in expression in UWM5, induced by glycerol, was very large indeed. One possible explanation was that a considerable degree of vegetative growth was still occurring on CF medium. If spores were only partially disrupted during sonication, then the assays for enzyme and total protein would detect an activity more characteristic of vegetative cells than of spores.

Another potential difficulty was the experimental variation in the recorded enzyme activities. This could account for the irreproducible results obtained with certain gene fusions, and also the variations observed when a gene fusion was transduced to a new strain. Such a difficulty can only be overcome by taking multiple samples at points on the timecourse rather than single ones. Even then, it may be

difficult to prove the existence of small changes in gene expression during development, as are encountered in some fusions such as UWM3.

Several additional sporulation induced gene fusions were isolated during subsequent experiments. However, these were of no particular interest in the present context as they showed even higher levels of induction when grown vegetatively on minimal medium. Such an observation, together with those summarised in Table 3-2 and Table 3-3 suggest that there is a much greater difference in global transcriptional activity when cells on rich medium are compared with sporulating cells than when cells on minimal medium are compared with sporulating cells. The latter comparison is the more significant, if the mechanism of sporulation is being investigated.

## CHAPTER 4

The effect of mutant genetic background in M. xanthus upon the expression of gene fusions

## 4-1 Introduction

In the previous chapter it was demonstrated how a fusion to the plasmid based promoter-probe could be cloned from the transductant in which it had been characterised. Such a plasmid could then be transduced to another strain. In the Introduction it was described how transposon generated gene fusions have been introduced into previously isolated sporulation defective mutants (Kroos L. et al. 1986), (Kuspa A. et al. 1986). This has allowed an epistasis map to be constructed showing the dependence of the promoter in each gene fusion upon the expression of a second gene of which a mutant allele is available.

This chapter deals with similar experiments involving the gene fusions obtained using the promoter-probe. The recipient strains are described more fully in Materials and Methods (2-1-3). Four different sporulation defective mutants were used: DK5057 and DK4727 from the A<sub>sg</sub> group, DK3621 from the D<sub>sg</sub> group and L5202 from the C<sub>sg</sub> group. The first three are apparently blocked at early stages of the developmental cycle, while the fourth one is blocked at a much later stage. In addition a nonmotile strain DK306 was used as a transduction recipient. This carries a mutant allele of the mgl locus and is consequently defective in both motility systems. Such a mutation renders the cells completely nonmotile and, consequently, unable to form fruiting bodies. This defect does not necessarily affect sporulation.

#### 4-2 Transduction of gene fusions into mutant strains

The cloned promoter-probe plasmid in E. coli was packaged in P1 and used to transduce different strains of M. xanthus to kanamycin resistance. The A9g and D9g strains were already kanamycin resistant owing to the presence of Tn5. Consequently the kanamycin resistance gene of Tn5 first had to be exchanged for the gene for oxytetracycline resistance by transduction with P1::Tn5-132 and selecting for tetracycline resistant, kanamycin sensitive transductants arising through double recombination. It was then possible to transduce these mutant strains with promoter-probe plasmids packaged with P1 phage.

#### 4-3 Assay conditions

Cells were grown in DCY medium then spun down and resuspended in TM before being spotted onto TM and A1 plates. For sporulation, TM was used in preference to CF medium as development proceeds more rapidly on this medium. To provide vegetative conditions as a control, A1 medium was used for reasons described in the previous chapter. Samples were taken of the cell suspension used for spotting onto plates in order to determine the specific enzyme activity at zero time. Samples were taken from both plates over several days. Several (usually four) samples were taken at a time from each plate.

With previous work on gene fusions (Kroos et al. 1986) it had been found that the expression of  $\beta$ -galactosidase was confined to spores. This possibility had therefore to be



considered here. Where samples contained spores the enzyme activity in spores was measured separately from that in the remaining vegetative cells. The distinction was possible because of the different sonication resistances of the two cell types (see Materials and Methods 2-14).

#### 4-4 Expression of isg-2>lacZ in different genetic backgrounds

Assays for  $\beta$ -galactosidase expression were carried out on mutant strains carrying the plasmid pUWM5 which contains the gene fusion isg-2>lacZ. The results are shown in Figs. 4-1-1 to 4-1-7 inclusive. In the wild type background (DK1622[pUWM5]) shown in Fig. 4-1-1, expression was actually slightly reduced from the level of expression in liquid culture, at least in the early stages of development. A similar reduction occurred in the control sample on A1 medium. However, there was an increase in expression within the spores, although this was less than twice the level of expression at the start of the experiment.

The situation was strikingly different when the gene fusion was present in the nonmotile strain DK306 (Fig. 4-1-2). Here the enzyme activity hardly changed at all in cells grown on minimal medium. There was, as before, a drop in expression in the remaining vegetative cells on the sporulation plate. However, a large number of spores were formed in spite of the absence of fruiting bodies. Within the spores there was a fifteenfold increase in  $\beta$ -galactosidase activity, although still not as great as that in glycerol induced spores (around

Fig. 4-1-1

Expression of *isg-2*lacZ during the development of DK1622[pUWM5]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Solid diamonds represent activity measured within spores. This activity was only released by prolonged sonication in the presence of 0.10-0.11mm glass beads.

Open triangles represent activity measured within control cells growing under vegetative conditions (on R1 medium).

Fig. 4-1-1

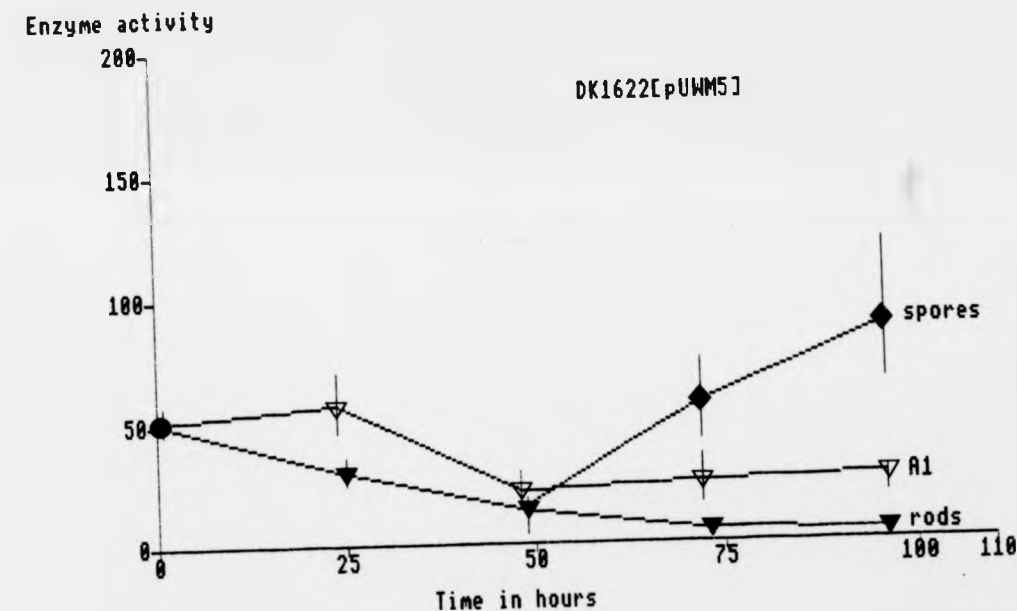


Fig. 4-1-2

Expression of *isg-2*lacZ during the development of DK306(Mg1)[pUW15]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Solid diamonds represent activity measured within spores. This activity was only released by prolonged sonication in the presence of 0.10-0.11mm glass beads.

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-1-2

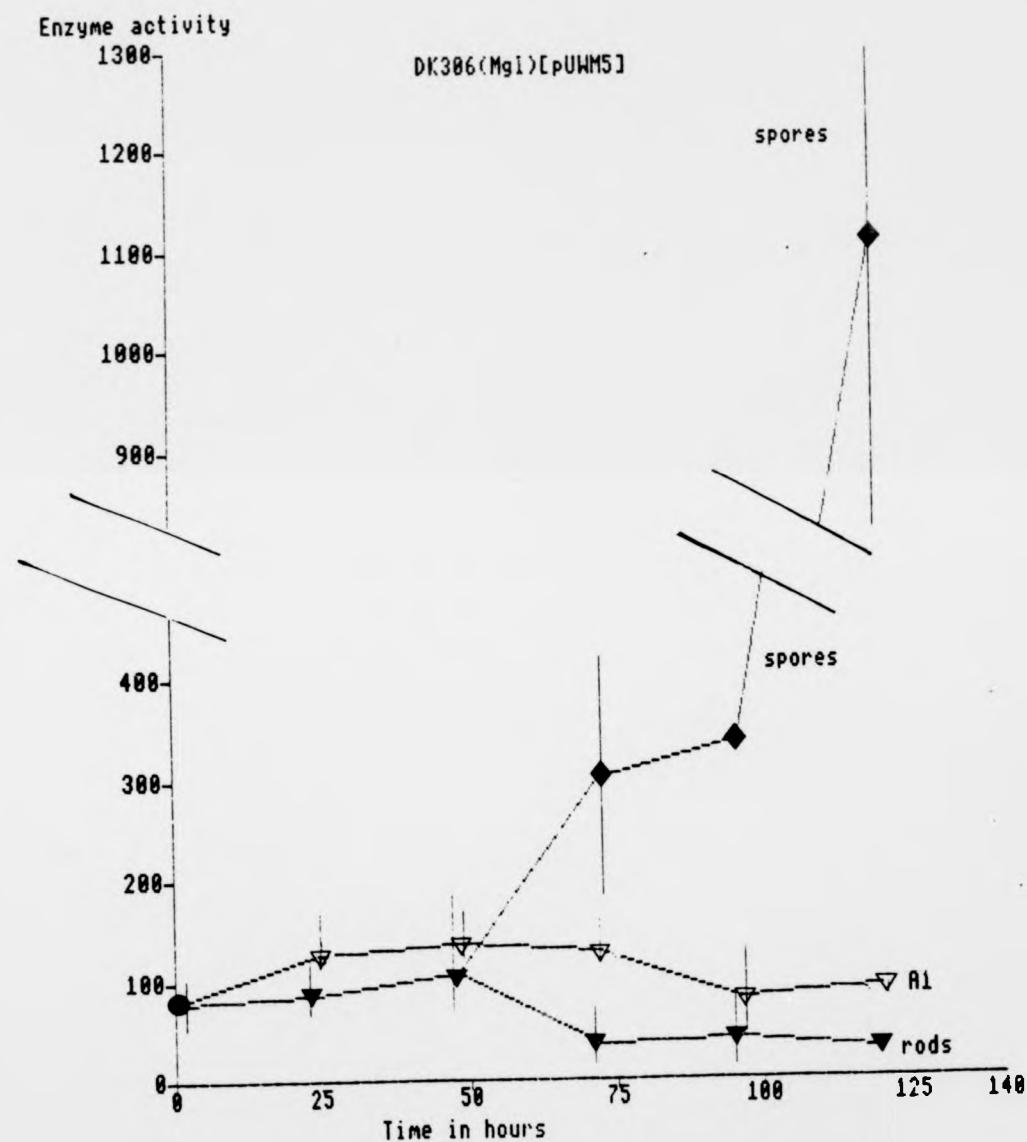


Fig. 4-1-3

Expression of *isg-2*lacZ during the development of DK5057(Asg)[pUWM5]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Fig. 4-1-4

Expression of *isg-2*lacZ during the development of DK3261(Dsg)[pUWM5]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-1-3

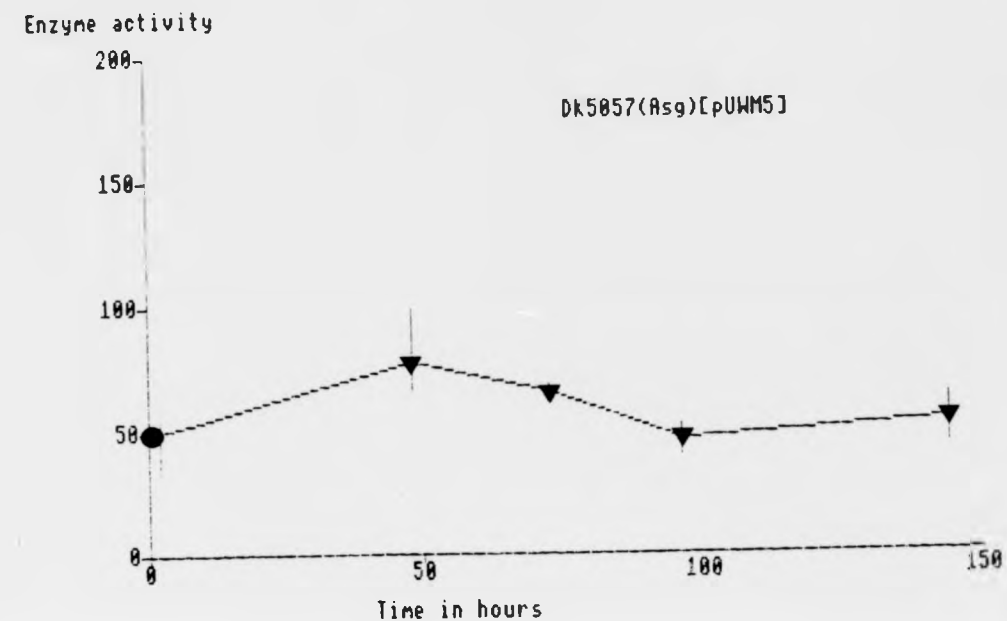


Fig. 4-1-4

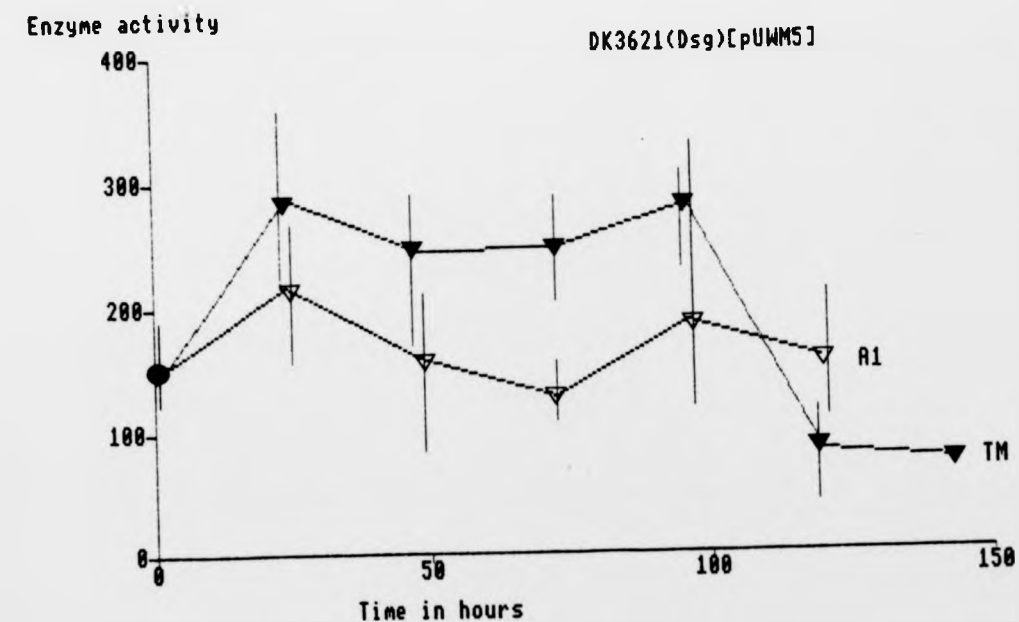


Fig. 4-1-5

Expression of *isg-2*lacZ during the development of LS202(Csg)[pUWM5::Tn5(-1300)]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Fig. 4-1-6

Expression of *isg-2*lacZ during the development of DK101[pUWM5]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Solid diamonds represent activity measured within spores. This activity was only released by prolonged sonication in the presence of 0.10-0.11mm glass beads.

Fig. 4-1-5

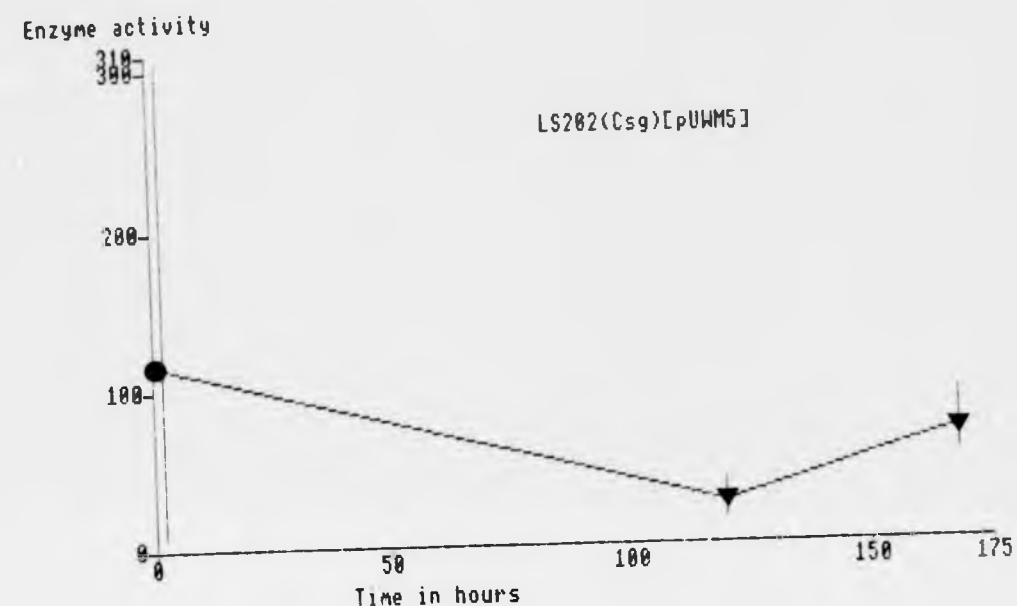


Fig. 4-1-6

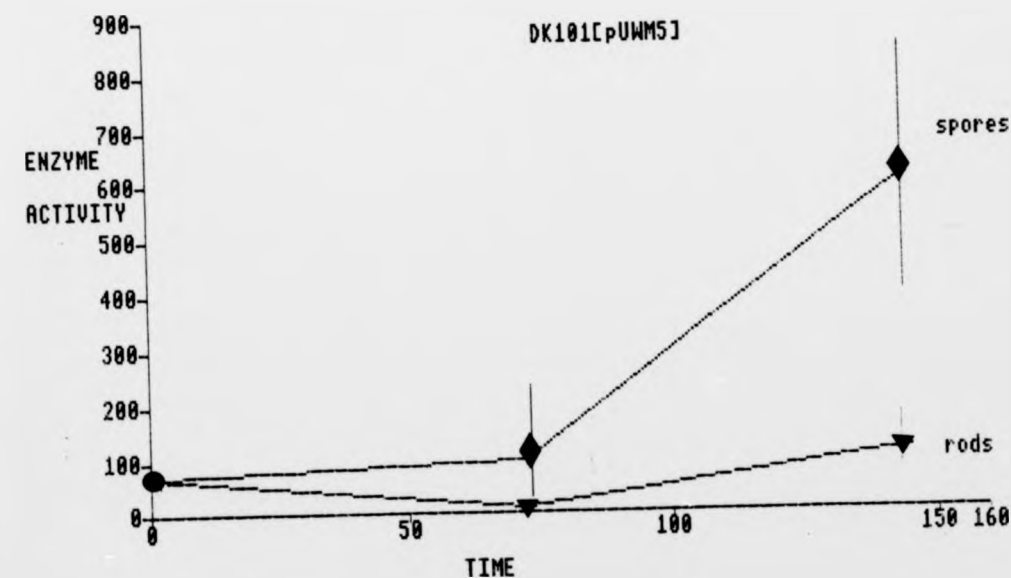


Fig. 4-1-7

Expression of  $lsg-2 \rightarrow lacZ$  during the development of strain UWMS

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

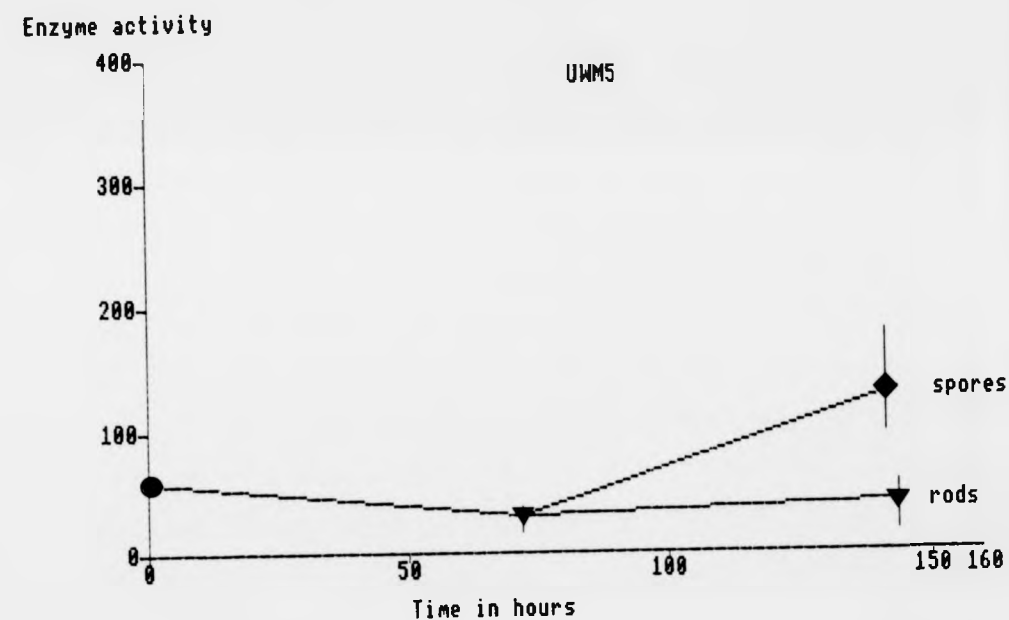
The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Solid diamonds represent activity measured within spores. This activity was only released by prolonged sonication in the presence of 0.10-0.11mm glass beads.

Fig. 4-1-7



eightyfold)(Fig. 3-8-2).

Enzyme activity was investigated in sporulation defective backgrounds placed under conditions which would normally induce sporulation. In a *Asg* group background there was a slight increase in activity, but only during days 2 and 3 (Fig. 4-1-3). In a *Dsg* group background the enzyme activity was significantly greater than in the other strains tested (Fig. 4-1-4). This was true at the outset of the experiment and expression increased still further in cells placed under sporulation conditions. There was, however a massive drop in expression on day 5. Where the plasmid was placed in a strain in the *Csg* group, observed expression remained virtually level (Fig. 4-1-5). However, in this experiment, no samples were taken between days 1 and 4 so the existence of an early peak in expression as with *Dsg* cannot be ruled out.

The gene fusion was also tested in the form of its original isolate UWMS which has an additional defect which prevents fruiting bodies from forming properly. It was also tested in a DK101 background where sporulation is delayed owing to the reduced functioning of the S motility system. Neither strain underwent development on TM medium but both did on TMP. As for expression of the fusion gene, the DK101[pUWMS] strain expressed the gene at an elevated level in spores (Fig. 4-1-6) although not at as high a level as DK306[pUWMS]. The strain UWMS, the original isolate containing the fusion showed scarcely any increase (Fig. 4-1-7).

All these findings are summarised as bar charts (Figs. 4-1-8 and 4-1-9). The initial surprise was that gene expression was hardly increased at all during sporulation in wild type cells (DK1622) and confirms the findings described



Fig. 4-1-8

Bar chart summarising *isg-2>lacZ* expression in different genetic backgrounds

The vertical axis represents enzyme activity in units per milligram of soluble protein. Activities are shown for a variety of strains carrying the gene fusion, both when in the vegetative state and when under sporulation conditions. The reading for vegetative expression (VE) is the average reading for cells harvested from rich medium in liquid culture. The reading for sporulation (SP) is the average reading for the day on which expression reached a maximum.

The average readings incorporate data shown in previous graphs and also other data not previously shown. The range of variation and the number of readings taken for each strain is shown in Fig. 4-1-9.

Fig. 4-1-9

Bar chart summarising *isg-2>lacZ* expression in different genetic backgrounds

This graph illustrates the range of variation encountered.

The vertical axis represents enzyme activity in units per milligram of soluble protein. Activities are shown for a variety of strains carrying the gene fusion, both when in the vegetative state and when under sporulation conditions. The maximum and minimum readings are shown for vegetative expression (V) and were obtained from cells harvested from rich medium in liquid culture. The maximum readings are shown as cross-hatched bars. The readings for sporulation (S) are the maximum and minimum readings for the day on which expression reached a maximum. Maximum readings are shown as unshaded bars. Minimum readings for both vegetative and sporulating conditions are shown darkly shaded. Data are incorporated both from previous graphs and from other data not previously shown. The number of readings taken for each strain (under both conditions) is shown above the bar for each maximum reading.

Fig. 4-1-8

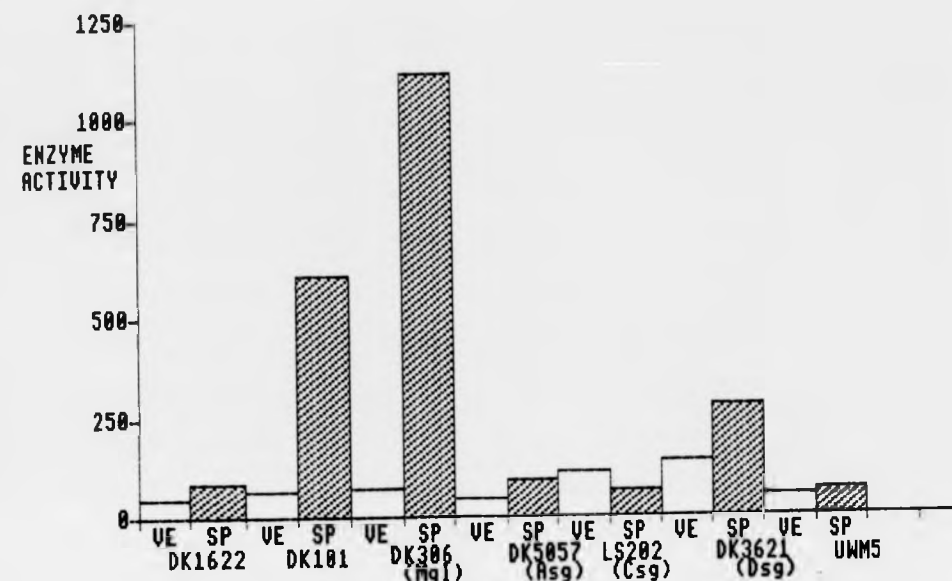
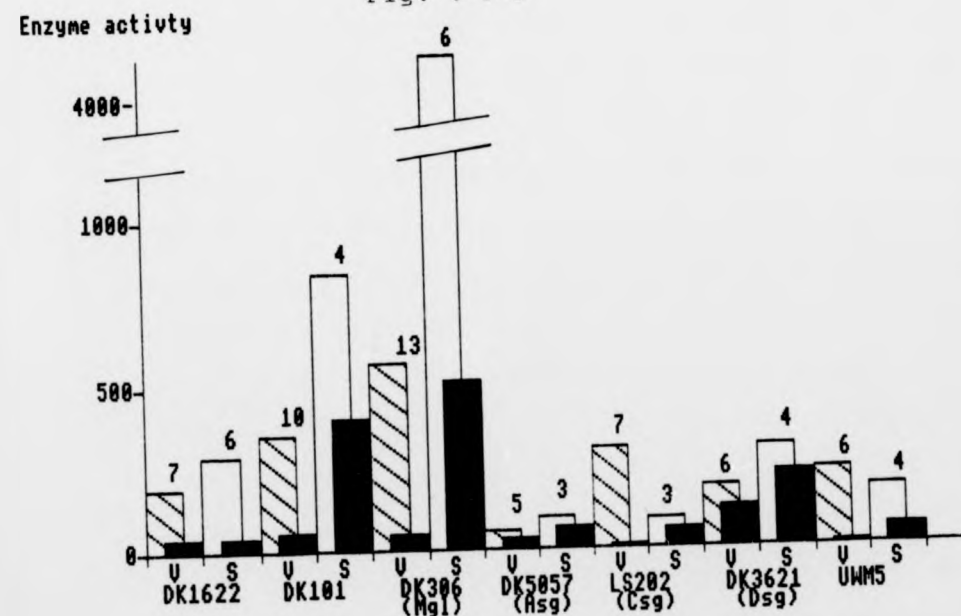


Fig. 4-1-9





in the previous chapter. This contrasts with the eightyfold increase in expression when sporulation is induced in liquid culture using glycerol or DMSO. The fact that gene expression was increased during sporulation in a nonmotile strain suggests that the gene in question is not expressed during normal sporulation but may have a function when spores are forced to form in the absence of fruiting bodies. The difference in expression has been demonstrated in a repeat of the experiment (Fig. 4-1-9).

In strain DK101, one of the two motility systems is reduced, although fruiting bodies can still form. Here the pattern of expression is intermediate between that of a fully motile strain and that of a nonmotile strain. However, the gene fusion shows little if any increase in expression in the original isolate which is a DK101 background plus an additional mutant allele which leads to aberrant fruiting bodies (see Chapter 3). The pattern of expression in a *Asg* background can be readily explained. The mutant is blocked early in sporulation and there is no significant increase in expression. The results for the *Dsg* mutant are different. Expression is significantly increased in this strain and the peak of expression is reached much earlier than would be reached in a sporulating strain (Fig. 4-1-4). It may be that the block in the sporulation process results in a gene normally expressed in spores being expressed in vegetative cells. It is known that the *Dsg* phenotype arises from a leaky mutation in an otherwise essential gene (Kaiser D. (unpublished results)). However, another possibility which cannot be excluded is that the plasmid integration was multimeric. This could have arisen if the plasmid packaged in

P1 for transduction had been multimeric. This would account for the observation of increased expression under both vegetative and starvation conditions. The level of expression of isg-2>lacZ observed in a Csg background decreased during the course of starvation. The Csg phenotype is blocked at a relatively late stage in sporulation (Kroos L. and Kaiser D. 1987). This suggests that the isg-2 gene is a gene expressed at a late stage of development. This correlates well with the observed time of expression in DK306 where the increase in expression is not apparent until 72 hours after the onset of starvation (Fig. 4-1-2).

#### 4-5 Expression of isg-1>lacZ in different genetic backgrounds

The plasmid PUWM4 containing the gene fusion isg-1>lacZ was transduced to several different strains of M. xanthus. Samples of vegetative and sporulating cells were harvested at intervals as described in the previous section and assayed for  $\beta$ -galactosidase and total protein. The data are shown in Figs. 4-2-1 to 4-2-4 inclusive. They are also summarised in a bar chart in Fig. 4-2-5. It is apparent that this gene fusion shows strongly increased expression not only during sporulation but also when growing vegetatively on A1 medium. This increased expression was found even in wild type cells grown in liquid A1 medium (Fig. 4-2-5). Although development cannot take place in liquid medium, the gene fusion increased its expression fivefold. This was comparable to the increase

Fig. 4-2-1

Expression of *isg-1*lacZ during the development of DK1622[pUWM4]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Solid diamonds represent activity measured within spores. This activity was only released by prolonged sonication in the presence of 0.10-0.11mm glass beads.

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-2-2

Expression of *isg-1*lacZ during the development of DK306(Mg1)[pUWM4]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-2-1

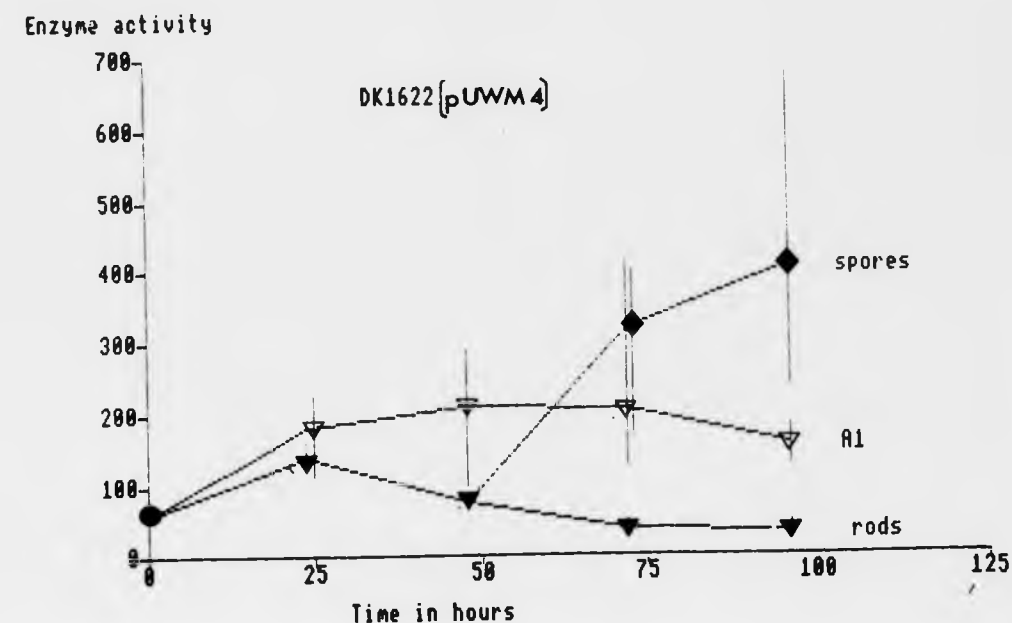


Fig. 4-2-2

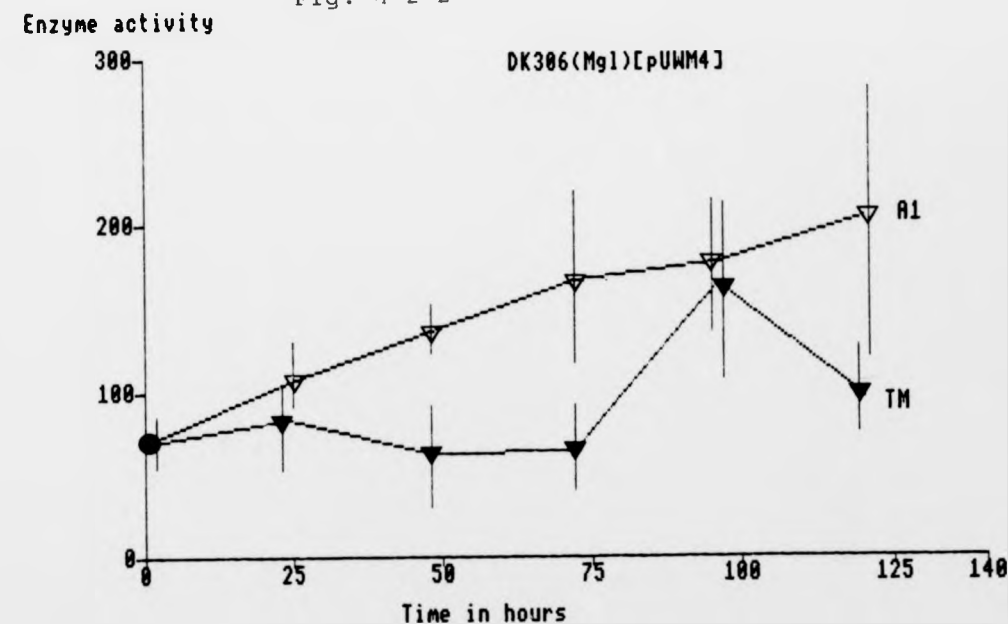


Fig. 4-2-3

Expression of *isg-1*lacZ during the development of DK5057(Asg)[pUWM4]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-2-4

Expression of *isg-1*lacZ during the development of DK3261(Dsg)[pUWM4]

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto TM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating.

The vertical bars represent the range of variation between the several (usually 4) samples taken at each point on the timecourse. The point on each bar indicates the average reading.

Solid triangles represent activity measured within rod shaped (vegetative) cells when under sporulating conditions (TM medium).

Open triangles represent activity measured within control cells growing under vegetative conditions (on A1 medium).

Fig. 4-2-3

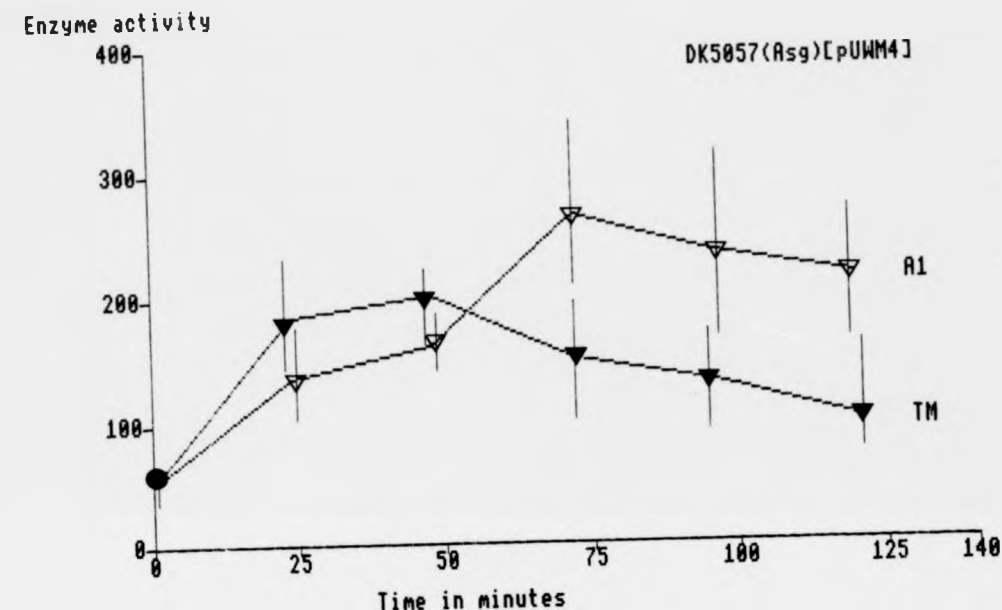


Fig. 4-2-4

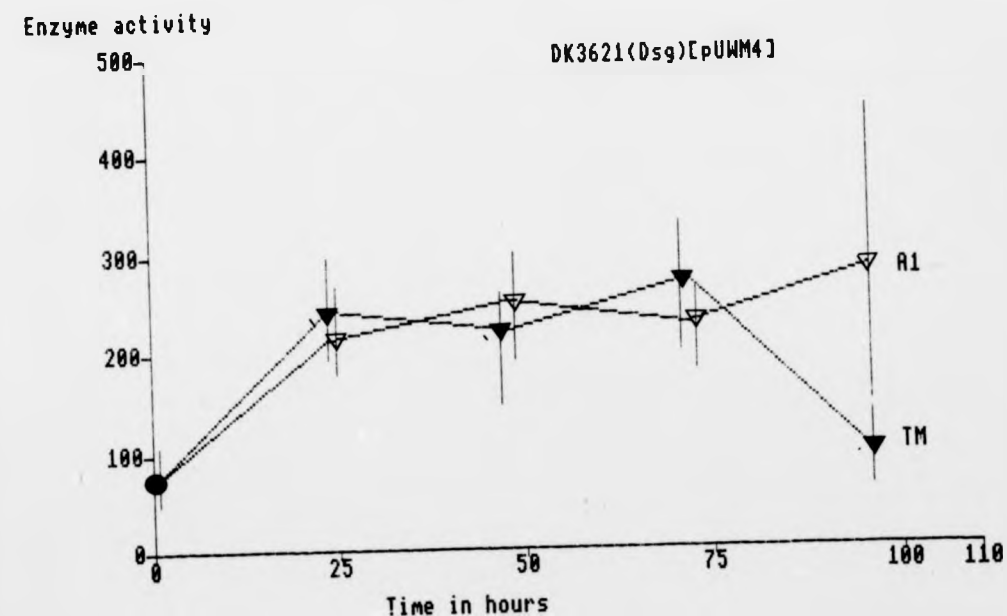


Fig. 4-2-5

Bar chart summarising *isg-1>lacZ* expression in different genetic backgrounds

The vertical axis represents enzyme activity in units per milligram of soluble protein. Activities are shown for a variety of strains, both when in the vegetative state and when under sporulation conditions. The reading for vegetative expression (VE) is the average reading for cells harvested from rich medium in liquid culture. The reading for sporulation (SP) is the average reading for the day on which expression reached a maximum.

The average readings incorporate data shown in previous graphs and also other data not previously shown.

For comparison, two extra bars are shown. One (R1) shows the expression in liquid minimal medium. The other (GLY) shows the level of expression obtained in spores induced by the addition of glycerol to cells growing exponentially in rich liquid medium (CTTYE).

Fig. 4-2-5



observed with sporulating cells. However there is also evidence the gene is involved in sporulation as well as during nutritional downshift. Induction of sporulation in rich medium, using glycerol, resulted in a fifteenfold increase in expression. Furthermore, in the case of strain DK1622[pUWM4] (Fig. 4-2-1), the gene was expressed preferentially in spores while undifferentiated cells in the same sample did not undergo an increase in expression.

The gene fusion was tested in the sporulation mutants DK5057 (Asg group) (Fig. 4-2-3) and DK3621 (Dsg group) (Fig. 4-2-4). It was also tested in a nonmotile background (DK306) (Fig. 4-2-2). In the case of DK306[pUWM4], spores were not obtained in sufficient quantity for assay. All strains showed an increase in expression over that in liquid CTTYE medium, on both A1 and TM medium. In the Asg and Dsg strains however, the peak levels of expression on TM were reached earlier than in the wild type and nonmotile strains. Expression of gene isg-1 does not require the expression of the AsgA and Dsg phenotypes. It would appear, therefore, that the gene is switched on early during development. However, it is also required later in development. The evidence for this would be the induction of the gene in glycerol induced spores. Here the requirement for Asg and Dsg is bypassed.

#### 4-6 Expression of pUWM2 in different genetic backgrounds

This gene fusion is expressed at an earlier stage of development than the other two. Comparisons were made of gene expression during the first 24 hours of development. The

original isolate was compared with both *Asg* strains and the *Dsg* strain. The results are displayed in Fig. 4-3. Samples were spotted onto TM medium only. As the observed increase in expression is relatively small, it was necessary, in some cases, to repeat the experiments to obtain further readings. The Student's T test was used to determine whether observed changes were statistically significant (Table 4-1). An increase in expression was shown to occur both in the original UWM2 isolate and also in the sporulating strain DK101 containing the gene fusion. The *Dsg* sporulation mutant was able to regulate expression of the gene fusion. However, in both *Asg* mutants, the rise in expression of the gene fusion was apparently blocked. Although in one of the strains (DK4727(asgB)[pUWM2]) there was a barely significant increase in expression during the course of sporulation, this was due to the unusually low levels of expression at time zero as compared with wild type strains. The sporulation level of expression did not differ from the vegetative levels measured in other mutant backgrounds or those measured in wild type backgrounds. The available data indicated that the vegetative level of expression in DK4727(asgB)[pUWM2] was lower than the corresponding level in strain UWM2 (Fig. 4-3). However, a T-test indicated that this difference was not statistically significant (95% probability) (Table 4-1).

#### 4-7 Concluding remarks

The large numbers of spores formed in the nonmotile strain DK306[pUWMS] was a surprising discovery. In a recent study



Fig. 4-3

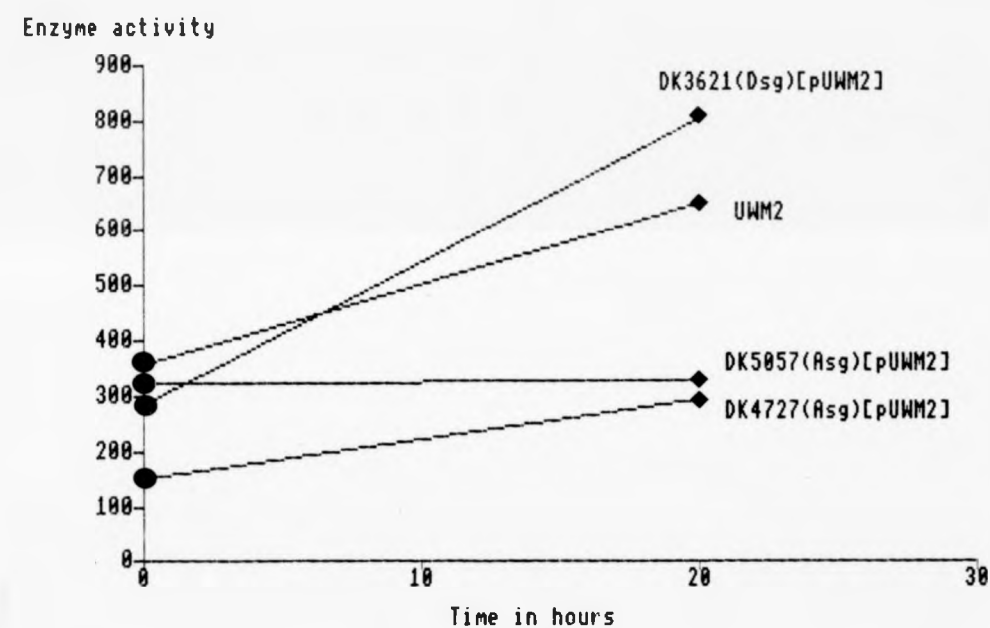
Sporulation regulated expression of  $\beta$ -galactosidase in *M. xanthus* strains carrying pUWM2

The vertical axis shows the activity of  $\beta$ -galactosidase in units per milligram of soluble protein. The units are described in Materials and Methods (2-14).

The horizontal axis shows the time in hours following plating onto IM medium. The point at time=0 (shown as a solid circle) was obtained from a sample taken from the broth culture before plating. A second point, at 20 hours is shown as a solid diamond. The line for UWM2 is the line already shown in Fig. 3-4-1.

A statistical analysis of the data shown in this graph is shown in Table 4-1

Fig. 4-3





Induction of  $\beta$ -galactosidase expression in strain UWM2 and in mutant strains containing the integrative plasmid PUWM2 (See also Fig. 4-3). However, additional data were used in this analysis. Data were available from a variety of experiments both of expression at zero time (i.e. vegetative expression) and also during the early stages (20-48 hours) of sporulation. Pairwise testing was carried out using the Student's T test (Bailey N.T.J. 1981) to determine whether there was a significant difference in expression levels between vegetative and sporulation conditions. Data for UWM2 (isolated in a DK101 background) and DK101[PUWM2] were combined in the columns headed "UWM2". Data obtained from DK101[PUWM2] are marked with asterisks.

In the case of DK4727(Asg)[PUWM2], the sporulation levels resembled the vegetative levels of other strains while the vegetative levels were unusually low. For this reason, additional pairwise testing was performed both sets this time against vegetative data for UWM5.

For the T test to be meaningful the two sets of data must be assumed to have similar standard deviations and both must be a normal distribution. The data for some gene fusions were gleaned from a variety of experiments performed on separate occasions. Their distribution cannot automatically be assumed to be normal.

Table 4-1

Strain UWM2:		Strain DK4727(Asg)[PUWM2]:	
vegetative	sporulating	vegetative	sporulating
213.0	907.3*	140.5	238.5
431.1	717.8*	227.9	413.6
468.6	121.4	143.8	221.5
340.8	106.5	132.9	215.1
319.5	681.6	109.3	208.7
53.2	777.4		236.4
46.8	852.0		276.9
247.7*	1067.1		553.8
434.5*	2023.5		296.3
Mean = 283.9	806.0	Mean = 150.9	
Standard deviation = 157.6	564.0	Standard deviation = 45.1	124.6
T = 2.674		T = 2.48	
Probability that such a difference could occur by chance is 0.0155		Probability that such a difference could occur by chance is 0.0308	
Strain DK5057(Asg)[PUWM2]:		Strain DK3621(Asg)[PUWM2]:	
vegetative	sporulating	vegetative	sporulating
474.9	247.1		
276.9	328.0		
404.7	304.6	345.1	643.3
210.9	285.4	366.4	660.3
242.8	349.5		651.78
	298.2		
	564.4		
	255.6		
Mean = 322.0	329.1	Mean = 355.71	
Standard deviation = 112.8	101.0	Standard deviation = 0.71	0.57
T = 0.117		T = 21.71	
Probability that such a difference could occur by chance is 0.909		Probability that such a difference could occur by chance is 0.00212	
Comparison of vegetative UWM2 with vegetative DK4727(Asg)[PUWM2]		Comparison of vegetative UWM2 with sporulating DK4727(Asg)[PUWM2]	
T = 1.82		T = 0.178	
Probability that such a difference could occur by chance is 0.094		Probability that such a difference could occur by chance is 0.861	

(Kroos et al. 1988), a nonmotile mgI<sup>-</sup> strain was found to convert at most, 1% of its cells to spores when spotted onto TMP agar. Although the experiment described in this chapter did not include the counting of spores, it was clear from the turbidity of the suspension after sonication in a cup horn sonicator, together with the observation of many phase bright spores under the microscope, that the proportion of spores was much greater than this. The same high levels of sporulation were observed in the parent nonmotile strain DK306. In both cases viable, heat resistant spores were produced. Therefore, the integrated plasmid was not responsible for the high levels of sporulation.

The observation that expression of isg-2 was increased enormously during development in some strains but not others, is evidence that alternative pathways are available during development in M. xanthus. Within wild-type fruiting bodies the gene is not switched on. However, the gene is switched on if sporulation is forced to occur in the absence of fruiting bodies. An unexpected result was obtained in a DK101 background which produces nearly normal fruiting bodies yet there is some activation of the gene fusion. By contrast, the original UWM5 strain (with a mutant UWM1000 background) produced highly aberrant fruiting bodies, yet the gene was not switched on within the spores. It is possible that the conditions determining whether or not the gene is switched on are more complicated than simply the presence or absence of fruiting bodies. Although sporulation in the absence of fruiting bodies is not regarded as a normal part of M. xanthus development it is frequently encountered in the related Stigmatella aurantiaca (Stephens K. and White D. 1990). In

this species fruiting bodies only form if starved  
stimulated either by light or by Stigmatella;  
neither are present no fruiting bodies appear  
appear in the undifferentiated cell swarm inst

It can be speculated upon, why there should be  
pathways for sporulation. In the natural environment  
sporulation is a response to conditions unfavorable for  
growth. Furthermore, the type of nutrient deficiency  
considers considerably as may its severity. A variety of  
pathways could, therefore, have evolved to cope with  
possibilities.

The gene fusion cloned in pUWM4 (isg-1) was  
always expressed in spores. However, the gene  
expressed in vegetative cells grown on minimal medium  
gene is expressed even in mutants known to be defective at an  
early stage in development. There is indeed a correlation  
between growth on minimal medium and the early stages of  
sporulation. Both are accompanied by an increase in  
levels (Mandil C. and Kaiser D. 1980). This is a  
characteristic of the prokaryotic stringent response.  
would appear to be expressed during the late stages of  
sporulation as well. Glycerol, which circumvents the  
requirements for the early stages in sporulation,  
induces a high level of expression of this gene.  
conclusion, the gene isg-1 appears to be expressed in the  
stages of development. Its induction cannot be blocked  
either by an early block in sporulation, such as the  
mutation or by preventing sporulation by making  
semistarved cells in liquid A1 medium.

The gene cloned in pUWM2 is not inducible

is therefore perhaps, not surprising that it is found to be induced at an early stage in sporulation. Glycerol appears to induce late sporulation genes only. It would not appear to be one of the very early genes either. Both the *Asg* mutants tested for epistatic effects upon this gene fusion, failed to show convincing evidence of developmental regulation of this gene.

## CHAPTER 5

Restriction mapping of the regions surrounding gene fusions pUWM4, pUWM5, A45 and D32, and attempts to clone the regions extending past the gene fusions

### 5-1 Introduction

A gene fusion contains only part of the transcription unit of interest. It was therefore desirable to clone the intact copy of the gene. To facilitate further studies on both the gene fusions and the intact genes, their restriction sites were mapped. Southern analysis was used to obtain a partial restriction map of the uncloned regions surrounding the fusion point in UWMS.

### 5-2 Cloning the chromosomal regions extending past the gene fusions

The almost entire sequence of the promoter probe plasmid is already known (Hodgson D.A. unpublished) and nearly all its restriction sites could be predicted. In order to clone the chromosomal copy of the gene identified by the gene fusion, it was necessary to use a restriction enzyme which cut within the vector but not within the insert (see Introduction Fig. 1-4). A selection of enzymes known to cut in the P1<sub>inc</sub> region were tested on the plasmids pUWM4, pUWM5, A45 and D32. None contained a site for EcoRV in the insert. Chromosomal DNA samples were prepared from M. xanthus containing the fusions and these were digested with this enzyme. The digests were size fractionated by agarose gel electrophoresis followed by electroelution of the region containing fragments greater than 11kb in length. This was necessary in order to exclude from

the cloning, fragments too small to contain both a plasmid origin and chromosomal sequences of greater length than those already cloned in the gene fusion (see Fig. 1-5). Recombinant clones were obtained in E. coli as described in Materials and Methods. Plasmid prepared from these was compared, by restriction analysis, with the corresponding gene fusion. One clone derived from the chromosomal DNA of fusion strain UWM5 was found where restriction sites matched those found in the cloned insert of the plasmid pUWM5. This was assumed to contain sequences cloned in the fusion plus additional material downstream and was named pUWM5' (Fig. 5-2-2). However, with strains UWM4 and D32, clones could not be obtained from an EcoRV digest of chromosomal DNA even after a second attempt. A clone was later successfully obtained from a PstI digest of chromosomal DNA of strain UWM4. As a PstI site lies within the ampicillin resistance gene of the plasmid, the new clone pUWM4' contains no P1inc sequences and does not confer ampicillin resistance (Fig 5-1-2). Similarly, a clone corresponding to pD32 was obtained from a PstI digest of chromosomal DNA containing the plasmid pD32. This contained additional chromosomal sequences in place of the lacZ gene. This was named pD32' (Fig. 5-3-2). In the case of fusion strain AY5; after initial failure, no further attempts were made to clone from chromosomal DNA.

5-3 Mapping of the cloned genes pUWM4, pUWM4', pUWM5, pUWM5', pD32 and pD32'.

A range of restriction enzymes was used to digest



Fig 5-1-1

## Restriction map of pUWM4

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the vector pUWM422 (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert. For clarity, restriction sites have not been shown on the vector in this diagram. The restriction sites in pUWM422 are shown in Fig. 5-5.

Restriction sites are abbreviated:

K	<u>K</u> pnI = <u>A</u> sp718
M	<u>M</u> luI
St	<u>S</u> tuI
X	<u>X</u> hoI

The following restriction sites were found not to occur in the insert:  
BamHI, ClaI, DraI, EcoRV, HindIII, PvuI, PvuII, SalI,  
ScaI, BglII, PstI

SmaI sites were very numerous and mapping was not attempted.

Fig. 5-1-1

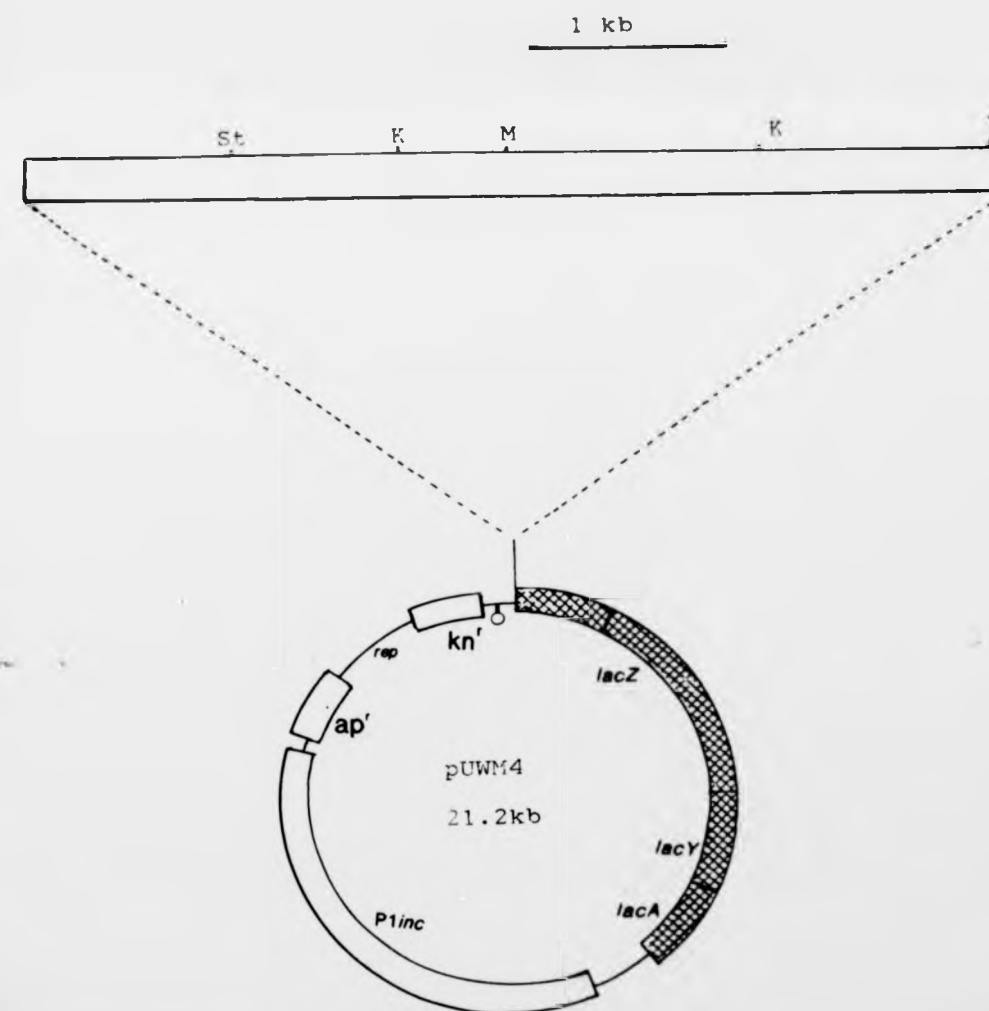




Fig 5-1-2

## Restriction map of pUWM4\*

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the remainder of the plasmid (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert.

The line on the linear map represents the point where the *lacZ* gene was fused in pUWM4. In this plasmid, however, *M. xanthus* DNA extends past this point.

Restriction sites are abbreviated:

Bg	<u>Bgl</u> II
H	<u>Hind</u> III
K	<u>Kpn</u> I = <u>Asp</u> 718
M	<u>Mlu</u> I
P	<u>Pst</u> I
Sc	<u>Sca</u> I
St	<u>Stu</u> I
X	<u>Xho</u> I

The following restriction sites were found not to occur in the insert:

BamHI, ClaI, NotI, EcoRV, HindIII, PvuI, PvuII, SalI.

SmaI sites were very numerous and mapping was not attempted.

Fig. 5-1-2

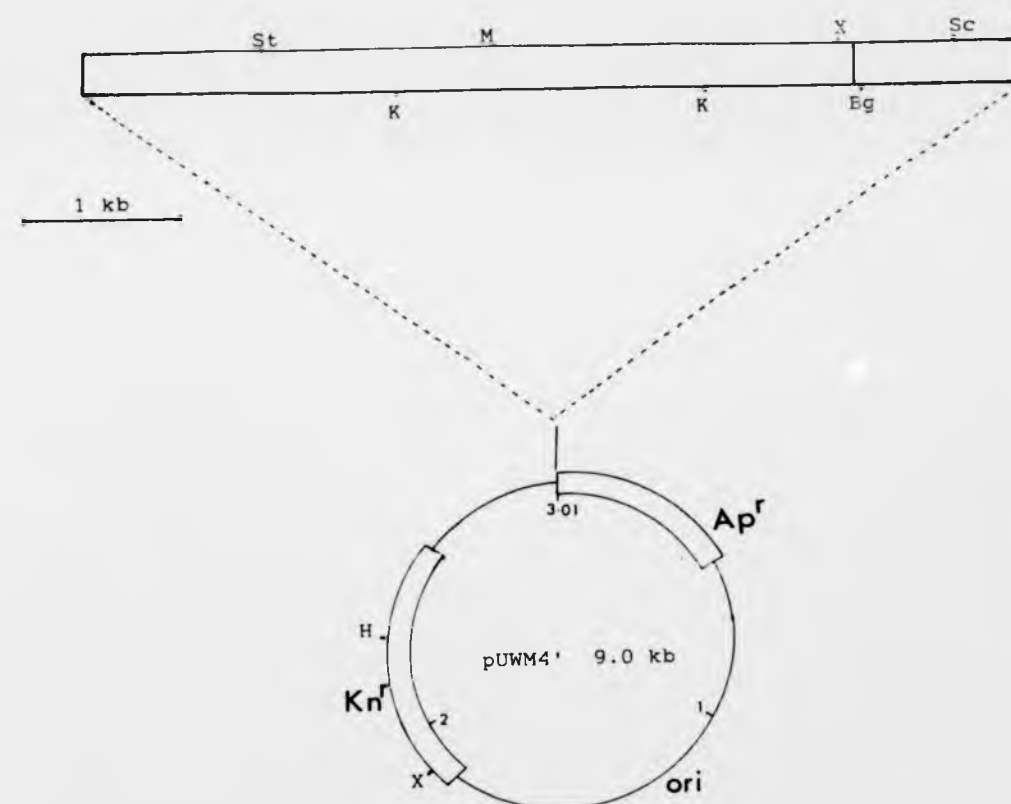


Fig 5-2-1

## Restriction map of pUWM5

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the vector pDAH122 (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert. For clarity, restriction sites have not been shown on the vector. The restriction sites in pDAH122 are shown in Fig. 5-5.

Restriction sites are abbreviated:

K	<u>Kpn</u> I = <u>Asp</u> 718
P	<u>Pvu</u> II
Ps	<u>Pst</u> I
Sa	<u>Sac</u> I
S	<u>Sma</u> I
St	<u>Stu</u> I
X	<u>Xho</u> I

The following restriction sites were found not to occur in the insert:

BamHI, BglII, DraI, HindIII, MluI, SalI, ClaI, EcoRV

Fig. 5-2-1

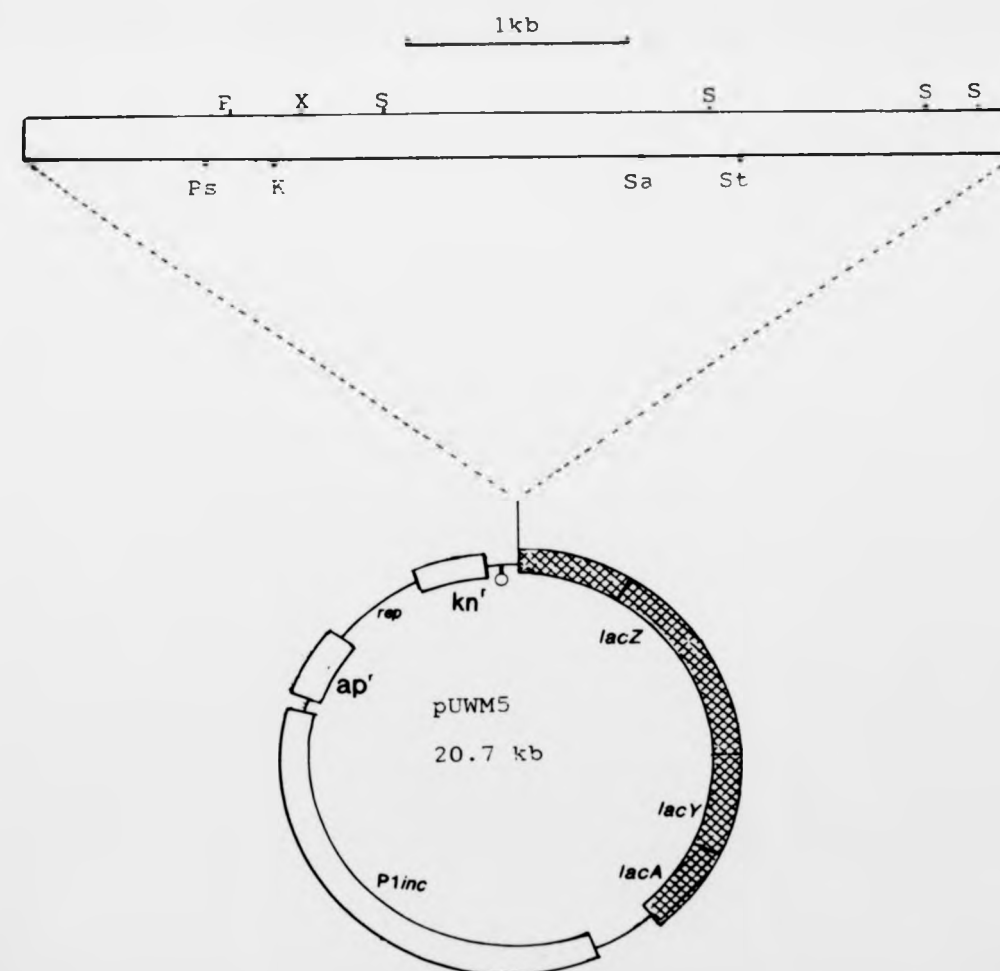


Fig 5-2-2

## Restriction map of pUWM5'

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the remainder of the plasmid (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert.

The line on the linear map represents the point where the *lacZ* gene was fused in pUWM5. In this plasmid, however, *M. xanthus* DNA was first thought to extend past this point. In fact the section of insert downstream of the fusion point was found to be part of the *lacZ* gene.

Restriction sites are abbreviated:

C	<u>Cla</u> I = <u>Bsc</u> I
E	<u>Eco</u> RV
H	<u>Hind</u> III
K	<u>Kpn</u> I = <u>Asp</u> 718
P	<u>Pvu</u> II
Ps	<u>Pst</u> I
Sa	<u>Sac</u> I
S	<u>Sma</u> I
St	<u>Stu</u> I
X	<u>Xho</u> I

The following restriction sites were found not to occur in the insert:  
BamHI, BglII, GraI, HindIII, HluI, SalI.

An EcoRI star site was found to occur just upstream of the fusion point.

Fig. 5-2-2

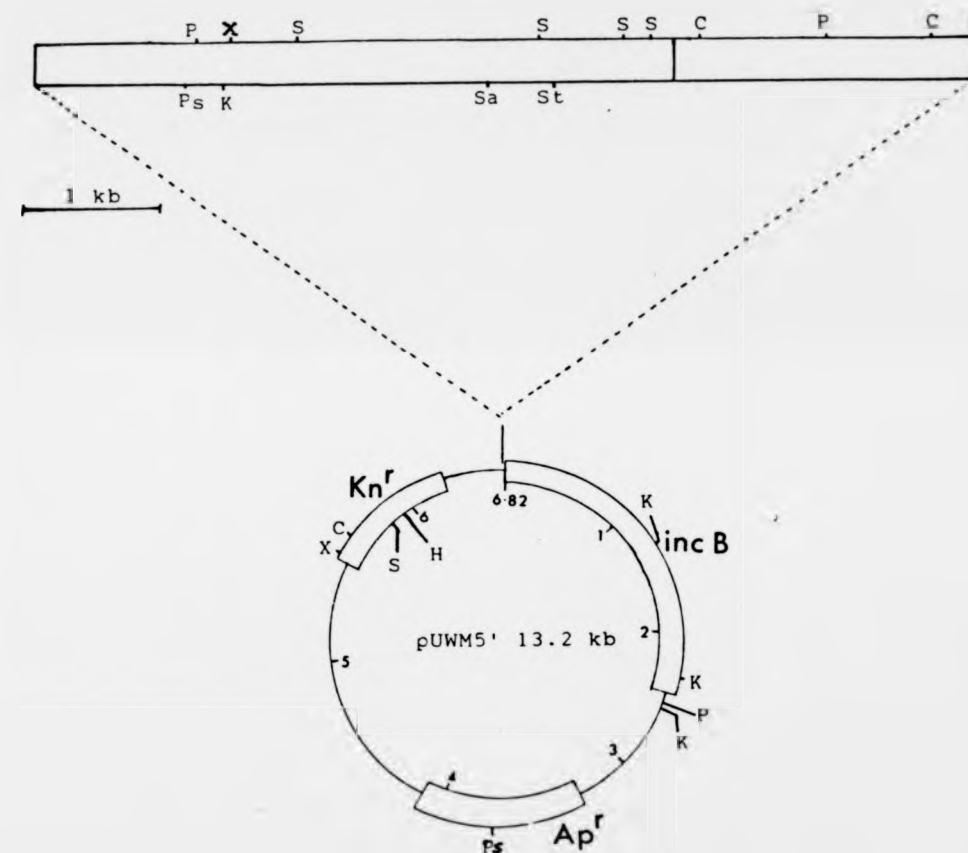


Fig 5-3-1

Restriction map of pD32

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the vector pDAH122 (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert. For clarity, restriction sites have not been shown on the vector. The restriction sites in pDAH122 are shown in Fig. 5-5.

Restriction sites are abbreviated:

M	<u>Mlu</u> I
P	<u>Pvu</u> II
R	<u>Eco</u> RI
St	<u>Stu</u> I
S	<u>Sma</u> I
X	<u>Xho</u> I

The following restriction sites were found not to occur in the insert:  
BamHI, ClaI, DraI, EcoRV, HindIII, KpnI, SalI,  
PstI

Fig. 5-3-1

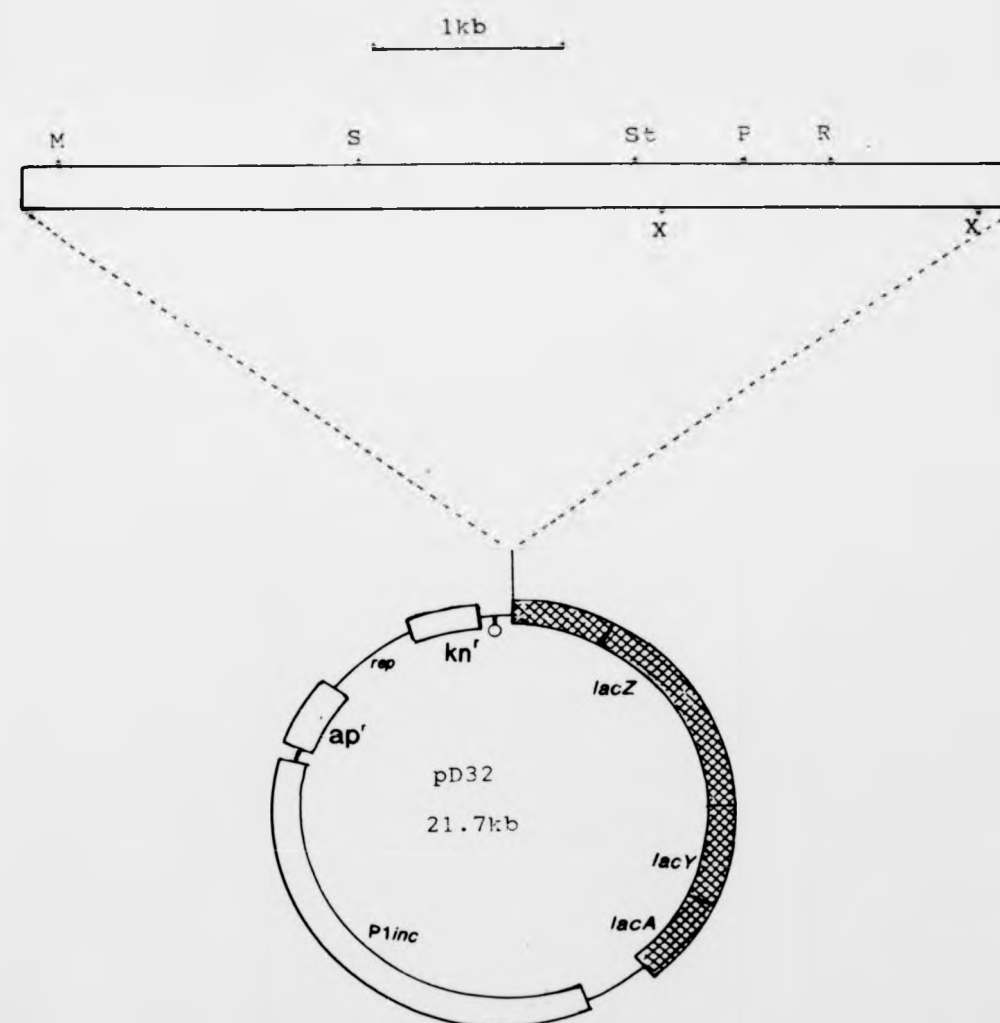


Fig 5-3-2

## Restriction map of pD32'

The insert of cloned *M. xanthus* DNA is shown as a linear map together with its point of insertion in the remainder of the plasmid (represented as a circle). Vector and insert are not to scale. The 1kb scale refers to the linear map of the insert.

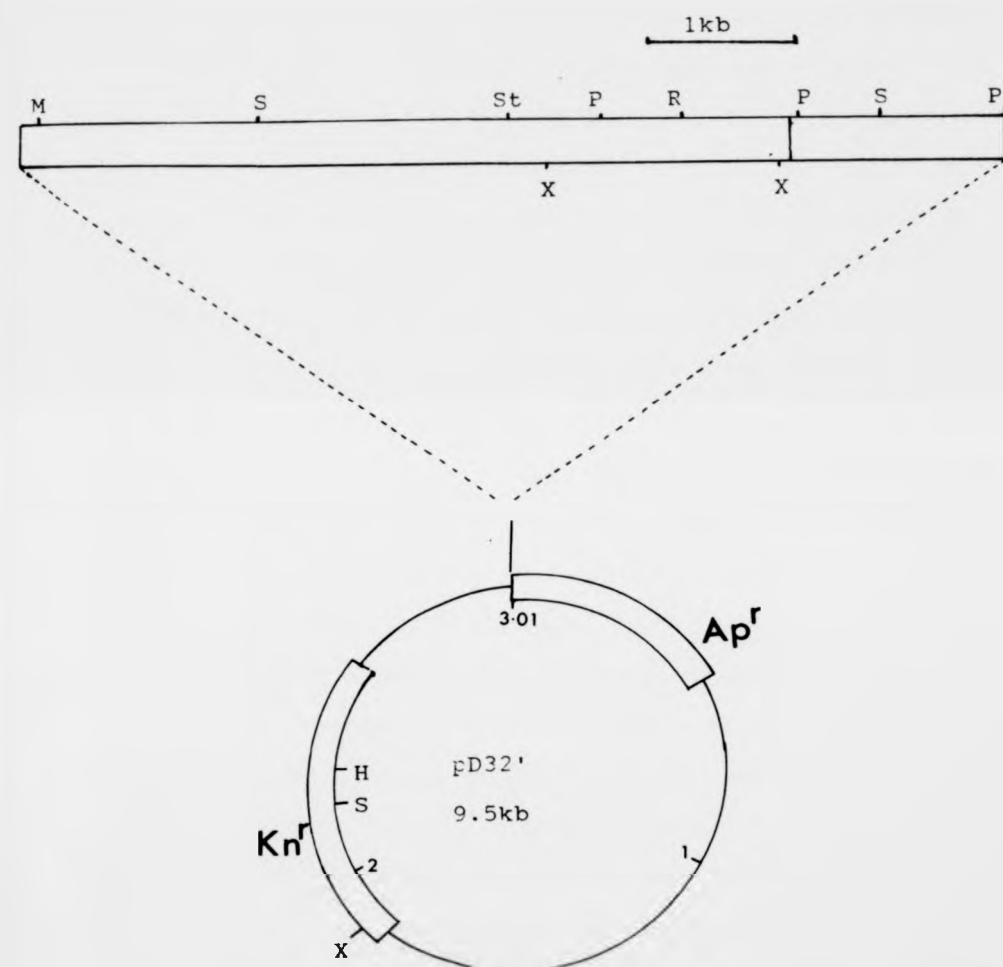
The line on the linear map represents the point where the *lacZ* gene was fused in pD32. In this plasmid, however, *M. xanthus* DNA extends past this point.

Restriction sites are abbreviated:

H	<u>H</u> indIII
M	<u>M</u> luI
P	<u>P</u> vuII
Ps	<u>P</u> stI
R	<u>E</u> coRI
St	<u>S</u> tuI
S	<u>S</u> maI
X	<u>X</u> hoI

The following restriction sites were found not to occur in the insert:  
BamHI, ClaI, GraI, EcoRV, HindIII, KpnI, SalI.

Fig. 5-3-2



plasmids: pUWM4; pUWM4'; pUWM5; pUWM5'; pD32 and pD32'. Where a restriction site occurred both in the vector and in the insert the site in the insert could be mapped. Where a site occurred in the insert but not in the vector it was necessary to perform a double digest with a second enzyme which was known to cut within the vector but not in the insert. Digests were separated on 0.5-0.6% agarose gels. A digest of a gene fusion such as pUWM5 would be run alongside the same digest of its chromosomal homologue (pUWM5'). The fragment sizes appearing on gels are shown in Tables 5-1-1, 5-1-2, 5-2-1, 5-2-2, 5-3-1 and 5-3-2. Certain sites proved difficult to map. The PvuII fragments in UWM5' were very similar in size and mapping was not possible at first. Only later after Tn5 mutagenesis of the plasmid was performed and the inserts mapped by restriction analysis (see Chapter 6) did it become possible to locate PvuII sites in the plasmid. The SmaI sites were also difficult to map. In this case it was due to their extraordinary abundance in some M. xanthus DNA. For example one SmaI fragment in pUWM5' was too small to visualise on a gel (about 200bp). However, it was assumed to exist on the basis of the size of the other fragments and also from the discovery of a Tn5 insertion (see Chapter 6) which did not alter the size of any of the other SmaI fragments obtainable from the plasmid. In the case of pUWM4 and pUWM4' there were so many SmaI fragments that their mapping was not possible. Maps of the plasmids pUWM4', pUWM5' and pD32' are shown in Figs. 5-1-2, 5-2-2 and 5-3-2 respectively. For comparison, maps of the gene fusion plasmids pUWM4 and pUWM 5 are shown in Figs. 5-1-1 and 5-1-2 and 5-1-3 respectively.

Subsequent work (see Chapter 6) revealed that the

Table 5-1-1

Fragment sizes released by digestion of plasmid pUWM4 using a variety of restriction enzymes and pairs of restriction enzymes

Sizes are in kilobase pairs

(d) denotes an assumed doublet band with the two bands indistinguishable on the gel.

(i) is a band assumed to be the result of an incomplete digestion.

(-) is a small fragment not discernible on the gel but whose existence is assumed from the sequence of the vector plasmid.

Enzyme:

<u>Hsp718/ HindIII</u>	<u>BscI</u>	<u>BglII/ HindIII</u>	<u>EcoRV</u>	<u>PvuI</u>
11.9	13	13	13	7.1
3.2	6.6	7.2	7.2	5.5
2.1	1.7			4.8
-				2.1
-				-
				-

Enzyme:

<u>SmaI</u>	<u>StuI/ HindIII</u>	<u>XhoI</u>	<u>XhoI/ SaiI</u>	<u>XhoI/ EcoRV</u>
14	9.5(d)	15	15	7.3
4.0(i)	2.2	6.6	6.6	6.9
2.2				5.4
1.9				2.1

The following enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

BglII, HindIII, PstI, ScaI.

Table S-1-2

Fragment sizes released by digestion of plasmid pUWM4\* using a variety of restriction enzymes and pairs of restriction enzymes:

Sizes are in kilobase pairs

\* Digestion with SmaI produced a banding pattern characteristic of a partial digest. Bands apparently resulting from partial digestion are not shown here. However, their sizes suggest that there are two more extremely small SmaI fragments not visible on the gel.

Enzyme:

<u>Asp718/</u> <u>HindIII</u>	<u>BglIII/</u> <u>HindIII</u>	<u>MluI/</u> <u>HindIII</u>	<u>ScaI/</u> <u>HindIII</u>
5.3	6.1	6.1	6.4
2.1	3.1	3.8	2.8
1.9			0.4

Enzyme:

<u>SmaI*</u>	<u>StuI/</u> <u>HindIII</u>	<u>XhoI</u>	<u>XhoI/</u> <u>SalI</u>
8	7.7	6.8	6.6
2.2	2.1	3.1	3.0
1.9			

The following enzymes and pairs of enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

ClaI, DraI, HindIII, PvuI, HindIII/PvuII, HindIII/EcoRI,  
BscI, PstI



Table 5-2-1

Fragment sizes released by digestion of plasmid PUWMS using a variety of restriction enzymes and pairs of restriction enzymes:

Sizes are in kilobase pairs.

Enzyme:

<u>Asp718</u>	<u>BscI</u>	<u>EcoRI/</u> <u>HindIII</u>	<u>EcoRV</u>	<u>PvuII</u>	<u>PstI</u>	<u>SacI</u>
12.7	11.7	12	14	5.6	15	13.3
5.3	5.9	8.5	7.1	4.9	4.3	4.3
1.1	1.6			4.2		
1.0				2.5		
				2.1		

Enzyme:

<u>ScaI/</u> <u>HindIII</u>	<u>SmaI</u>	<u>StuI/</u> <u>HindIII</u>	<u>XhoI</u>	<u>XhoI/</u> <u>SalI</u>	<u>XhoI</u> <u>EcoRV</u>	<u>MluI</u>
15	13.3	10	14.6	15	7.3	12.2
2.3	3.1	7.0	3.0	3.0	5.4	5.0
	1.6	4.6			5.1	
					4.0	

Enzyme:

PvuI

6.4  
5.3  
4.8

The following enzymes and pairs of enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

BglII, BamHI/HindIII, HindIII

Table 5-2-2

Fragment sizes released by digestion of plasmid pUWMS' using a variety of restriction enzymes and pairs of restriction enzymes:

Sizes are in kilobase pairs.

Enzyme:

<u>Asp718</u>	<u>BscI</u>	<u>PvuI</u>	<u>PvuII</u>	<u>PstI</u>
6.8	5.9	6.6	4.9	9.2
5.3	5.7	4.0	4.3	4.3
	1.6	2.1	3.5	

Enzyme:

<u>ScaI/ HindIII</u>	<u>SmaI</u>	<u>StuI</u>	<u>XhoI</u>	<u>XhoI/ SalI</u>
10	7.9	9.1	10.0	10
2.3	3.1	4.2	3.0	3.0
	1.7			

The following enzymes and pairs of enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

BamHI/HindIII, MluI, HindIII, SacI

Table 5-3-1

Fragment sizes released by digestion of plasmid pD32 using a variety of restriction enzymes and pairs of restriction enzymes:

Sizes are in kilobase pairs.

(-) is a small fragment not discernible on the gel but whose existence is assumed from the sequence of the vector plasmid.

Enzyme:

<u>Asp718/</u> <u>HindIII</u>	<u>BscI</u>	<u>HindIII/</u> <u>EcoRI</u>	<u>PvuII</u>	<u>EcoRV</u>
16	14	14	5.3	15
3.3	6.5	5.3	5.2	7.1
1.0	-	4.1	2.5	
			1.7	
			-	
			-	

Enzyme:

<u>ScaI/</u> <u>HindIII</u>	<u>SmaI</u>	<u>StuI/</u> <u>HindIII</u>	<u>XhoI</u>	<u>XhoI/</u> <u>SalI</u>
19	18	10.0	16	15
2.4	3.5	9.0	5.3	5.3
		4.2	1.5	1.6

The following enzymes and pairs of enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

BglII, HindIII, PstI

Table 5-3-2

Fragment sizes released by digestion of plasmid pD32<sup>+</sup> using a variety of restriction enzymes and pairs of restriction enzymes:

(d) denotes an assumed doublet band with the two bands indistinguishable on the gel.

Sizes are in kilobase pairs.

Enzyme:

<u>HindIII/</u> <u>EcoRI</u>	<u>PvuII/</u> <u>HindIII</u>	<u>MluI/</u> <u>HindIII</u>	<u>SmaI</u>
5.3	5.2	8.2	4.0
4.2	2.9	1.1	3.5(d)
	1.3		

Enzyme:

<u>StuI/</u> <u>HindIII</u>	<u>XhoI</u>	<u>XhoI/</u> <u>SalI</u>
4.2	5.3	5.3
3.7	3.3	3.3
2.4	1.4	1.4

The following enzymes and pairs of enzymes were tested, and found to leave a single fragment corresponding to the linearised plasmid:

Asp718, HindIII, HindIII/BglII, DraI, PvuI,  
BscI HindIII/ScaI

sequences in pUWMS' downstream of the fusion point could not possibly be cloned M. xanthus DNA. An examination of the restriction sites revealed that the region was, in fact, part of the lacZ gene extending up to its EcoRV site. In view of this, several more attempts were made to clone the chromosomal homologue of the gene fusion. Chromosomal DNA from M. xanthus strain UWMS was digested using ScaI, BglII and MluI then circularised by ligation and used to transform E. coli. These attempts were without success. In no case could the chromosomal extension of the gene already cloned as a gene fusion be cloned.

#### 5-4 Southern analysis of the isg-2 region

The gene fusion obtained in strain UWMS (gene fusion isg-2) is of particular interest due to its increased expression during glycerol induced sporulation and also, under certain circumstances, during developmental sporulation. The only restriction mapping so far carried out had been on the 4.3kb upstream of the fusion point, the region which had already been cloned in pUWMS. The chromosomal homologue of the gene, which would include additional sequences downstream of the fusion point had not been successfully cloned. For this reason it was decided to carry out further mapping of the region by Southern analysis. Once a map of restriction sites in the chromosomal region flanking the cloned sequences is available, cloning the region becomes technically simpler. It also becomes easier to map the positions of any transposon insertions obtained by site directed mutagenesis of the gene

(Chapters 6 and 7).

Chromosomal DNA from the wild type strain DK1622 was digested with a variety of restriction enzymes known to cut the cloned insert in pUWM5. Southern analysis using the cloned insert as a probe should reveal a pair of bands in each track. Both bands will represent fragments with one end at a known site in the cloned region and the other end in one of the flanking regions. There were several restriction enzymes which did not have sites in the cloned insert but did have sites in Tn5. By this stage, *M. xanthus* strains had been created containing this transposon in the cloned region (Chapter 6). DNA was prepared from a strain derived from DK1622 containing Tn5 inserted at a point 800bp upstream of the point where the fusion exists in the gene fusion pUWM5'. The DNA was digested with a range of enzymes. These digests, together with the digests of DK1622 DNA were run on an agarose gel. A Southern blot was made of the gel. The nitrocellulose was probed with pUWM5'. From the positions of the bands on this and the calculated fragment sizes (Table 5-4 and Fig. 5-4-1), it was possible to determine the approximate positions of restriction sites outside of the cloned region (Fig. 5-4-2).

In the instances where a restriction site was known to be close to the end of the cloned region, the bands on the Southern differed considerably in intensity (Fig. 5-4-1). The transfer efficiency of DNA fragments did not appear to be dependent upon size (Fig. 5-4-1). This enabled the two bands to be correctly identified with the two fragments containing sequences from the cloned region. The PvuII sites could be identified in this way. In the case of the HindIII digests, one band was extremely faint. The position of the site was,

Table 5-4

Sizes of DNA fragments revealed on a Southern blot by hybridisation with pUWM5':

Sizes are in kilobase pairs.

(f) denotes an extremely faint band presumably due to poor digestion.

Chromosomal DNA from DK1622:

Enzyme:

<u>Asp718</u>	<u>PvuII</u>	<u>PstI</u>	<u>XhoI</u>
9.3	4.4	4.6	6.6
5.0	3.4	1.4	1.4

Chromosomal DNA from DK1622[85-800]:

Enzyme:

<u>BamHI</u>	<u>BglII</u>	<u>HindIII</u>	<u>EcoRV/ NcoI</u>	<u>MluI/ HindIII</u>
10.9	14.7	22.6	13.5	17.0
4.7		8.1(f)	3.5	8.1

<u>NcoI</u>	<u>ScaI/ BamHI</u>
13.7	10.8
3.5	8.1
	4.7

Fig. 5-4-1

Southern analysis of the lsq-2 region

The Southern blot was probed with pUWM5'

The positions of lambda size markers are shown as calculated from their position on the original gel (not shown).

Track:	Enzyme:	DNA:
1	<u>PvuII</u>	OK1622
2	<u>KpnI</u>	"
3	<u>PstI</u>	"
4	<u>XhoI</u>	"
5	<u>BglII</u>	OK1622[25-800]
6	<u>NcoI</u>	"
7	<u>BamHI</u>	"
8	<u>HindIII</u>	"
9	<u>ScaI/BamHI</u>	"
10	<u>MluI/HindIII</u>	"
11	<u>EcoRV/NcoI</u>	"

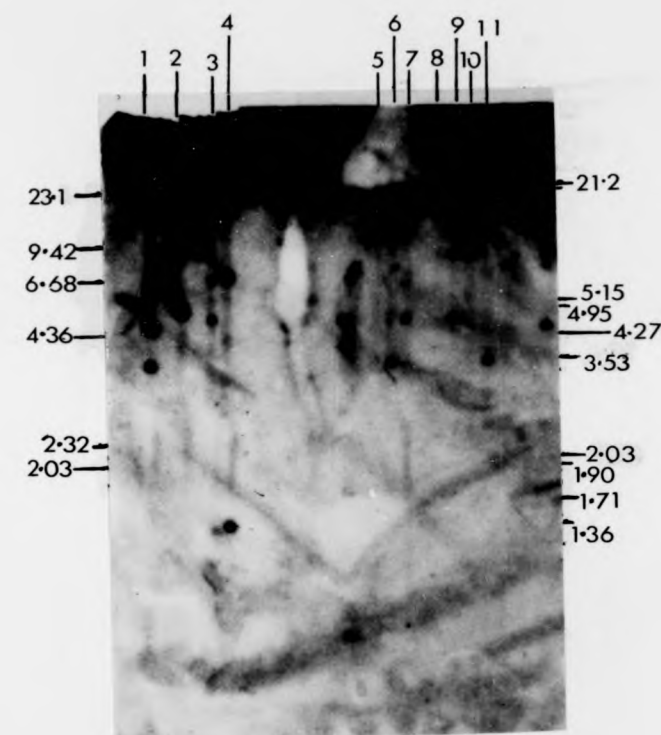




Fig. 5-4-2

Map of the *isg-2* region obtained by Southern analysis

The insert of *Tn5* is shown as it has integrated in strain DK1622[55-800].

An arrow beneath the map indicates the extent of *M. xanthus* DNA already cloned in pUW15.

The small arrow above the map indicates the direction of transcription of the *isg-2* promoter already characterised as a fusion to *lacZ*.

The identification of restriction sites outside of the cloned region is inevitably incomplete. Only those restriction fragments hybridising to the cloned region can be identified by Southern analysis. Not more than two sites from outside of the cloned region can ever be identified.

Restriction sites are abbreviated:

B	<u>Bam</u> HI
Bg	<u>Bgl</u> II
H	<u>Hind</u> III
K	<u>kpn</u> I = <u>hsp</u> 710
M	<u>Mlu</u> I
N	<u>Nco</u> I
P	<u>Pvu</u> II
Ps	<u>Pst</u> I
S	<u>Sal</u> I
Sa	<u>Sac</u> I
Sc	<u>Stu</u> I
X	<u>Xho</u> I

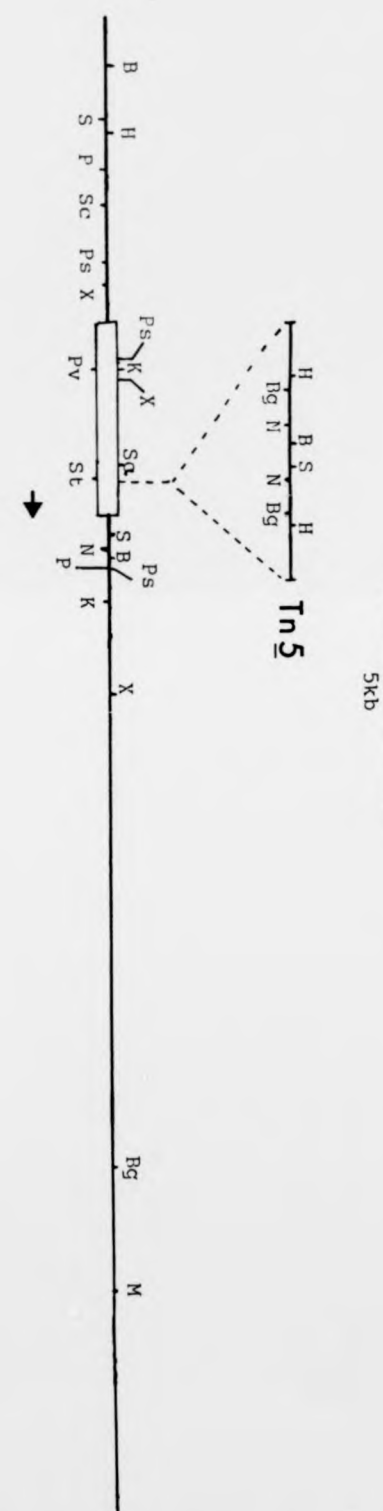
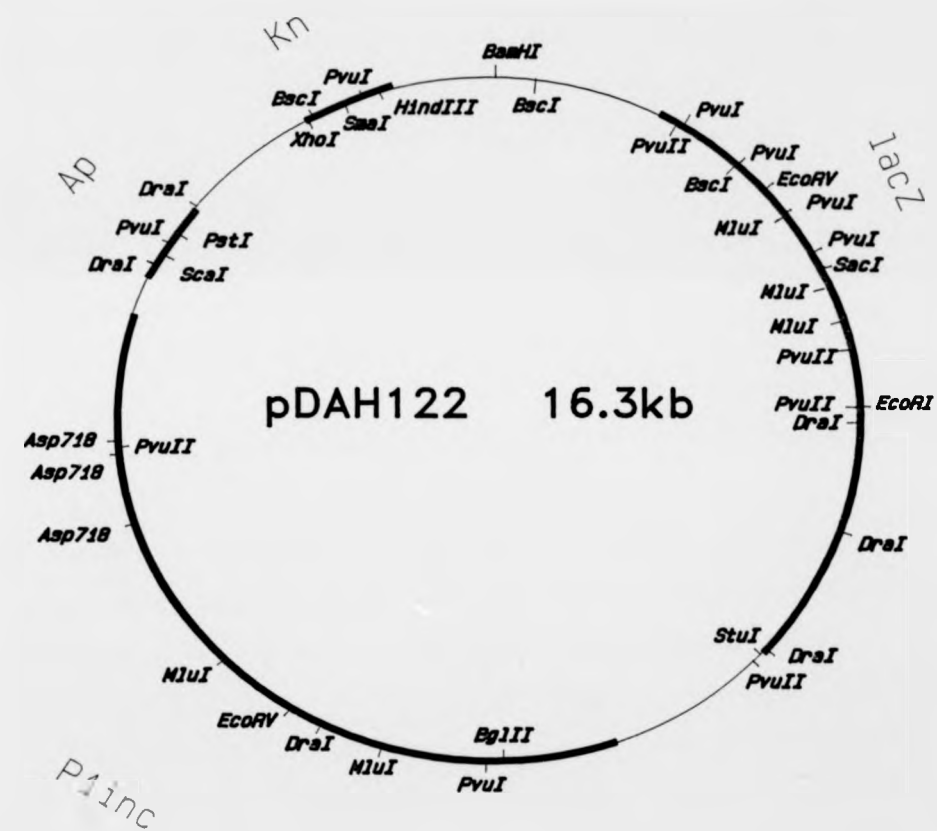


Fig. 5-4-2

Fig. 5-5

Restriction map of plasmid pDAH122

Fig 5-5



however, corroborated by experiments described in Chapter 7 (Figs. 7-7 and 7-9). The BglII site was very hard to define. Only one band was visible on the Southern blot. From comparison with band intensities of bands on other tracks, it is more likely to represent a 800bp hybridising region than a 3500bp hybridising region. In other instances the fragments could be correctly identified on the basis of their size alone. For example, the smaller BamHI fragment revealed on the Southern blot was about 5.5kb in length. In this instance, a BamHI site was present in a Tn5 insert within the cloned region. It is known that neither the cloned region nor Tn5 contain any other BamHI sites. Therefore the 5.5kb fragment must extend in a rightwards direction from the BamHI site in the transposon.

#### 5-5 Concluding remarks

Attempts were made to clone the chromosomal regions containing the cloned segments. Of four gene fusions, two were successfully cloned. A third clone pUWMS' was initially thought to contain the chromosomal homologue of the gene in pUWMS. However, subsequent findings revealed that the clone was simply the gene fusion cloned in the promoter probe (pUWMS) which had subsequently undergone an internal deletion. In fact pUWMS' is a recircularised form of the larger of the two EcoRV fragments derived from pUWMS. This fact has subsequently been confirmed by ligating in an EcoRV fragment from the promoter probe plasmid pDAH122 to regenerate a complete lacZ gene (Chapter 7). Presumably, when the clone was

obtained from chromosomal DNA of strain UWMS, closed circular DNA was present as well. When the DNA was digested with EcoRV, size fractionated and ligated, a portion of the plasmid was cloned instead of a chromosomal fragment as intended.

Subsequent attempts to clone the chromosomal homologue were not successful. This may be because there were no nearby sites in the chromosome for the enzymes used. For example although MluI was used in an attempt to clone the gene it is clear, from the results obtained by Southern blotting, that the nearest downstream site was nearly 20kb in distance from the site in the intact gene corresponding to the fusion point. There is no theoretical limit to the size of a plasmid obtainable through recircularisation of chromosomal DNA fragments. It is true that very large plasmids transform E. coli with lower efficiency. However, the difference in transformation efficiency is at most fivefold (Hanahan D. 1983). Another possible explanation might be that the gene cannot be cloned because its expression is lethal to E. coli. This can a problem when cloning certain prokaryote genes in E. coli. These include genes for surface structural proteins such as ompA in E. coli (Beck E. and Bremer E. 1980) and genes for enzymes important in cell metabolism such as polA in E. coli (Murray N.E. and Kelley W.S. 1979).

The restriction mapping of the isg-1 and isg-2 regions showed that these were different DNA sequences. Consequently, they can be given separate gene abbreviations as well as separate allele numbers. In subsequent chapters, the genes will be referred to as isgA1 and isgB2 respectively.

## CHAPTER 6

Targeted mutagenesis of  
the isgA1 and isgB2 genes and  
their surrounding regions

## 6-1 Introduction

Gene fusions in M. xanthus have been isolated which are induced in vivo during sporulation (Chapters 3 and 4). In some cases, adjacent sequences have been cloned from the fusion containing strains (Chapter 5). In the case of the gene fusion isolated as PUWM5 (lsgB2), the adjacent regions have been mapped by Southern analysis. The function of the genes, for which developmentally regulated gene fusions have been isolated, remains unknown. In Chapter 1 it was described how an integrative promoter-probe plasmid integrates into the chromosome to produce a gene fusion, and that an additional, complete copy of the same gene remains in the chromosome (Fig. 1-4). For this reason, the creation of the gene fusion should not be expected to be mutagenic. This, indeed, appeared to be the case. Only one of the gene fusions characterised, showed any obvious mutant phenotype. In this case (PUWM5) it was found to be the result of a second, unlinked mutation. However, the creation of a mutant allele with an altered phenotype can help to elucidate the function of a gene. Consequently, the cloned genes already obtained were used in the site directed mutagenesis of M. xanthus. Inserts of transposon Tn5 were obtained in the plasmids PUWM4' and PUWM5'. Such constructs could then be used to create mutants in DK101 or DK1622 by gene replacement.

6-2 The principle of Tn5 mutagenesis

The transposon Tn5 is a broad host range transposon. It contains a kanamycin resistance marker. It also has genes for streptomycin and bleomycin resistance, although streptomycin resistance is only expressed in certain host species. The transposon can replicate by inserting new copies of itself at other sites in the genome. There is, apparently, a nearly random choice of sites in the genome for insertion. Insertions can equally well occur in the chromosome or in a plasmid. Transposon Tn5 can transpose in M. xanthus and has been used extensively in the study of this species (Chapter 1). However, for the site-directed mutagenesis described in this chapter, the mutagenesis was carried out in E. coli, using as a target the cloned M. xanthus DNA. In this way, transposon insertions are obtained solely in the region of interest in the M. xanthus chromosome. Kanamycin resistance in E. coli is dependent upon gene dosage. Using sufficiently high levels of kanamycin, it is possible to select for insertions in a high copy number plasmid as opposed to insertions in the chromosome. Restriction mapping of the plasmids with inserts, should reveal which inserts occur within cloned M. xanthus DNA. Such inserts can be used in marker exchange procedures to generate Tn5 insertion mutants. To achieve this, the mutated plasmid is packaged in P1 phage and used to transduce M. xanthus (Chapter 3).

There are several possible outcomes for homologous sequences containing Tn5, once transduced into M. xanthus

## 6-1

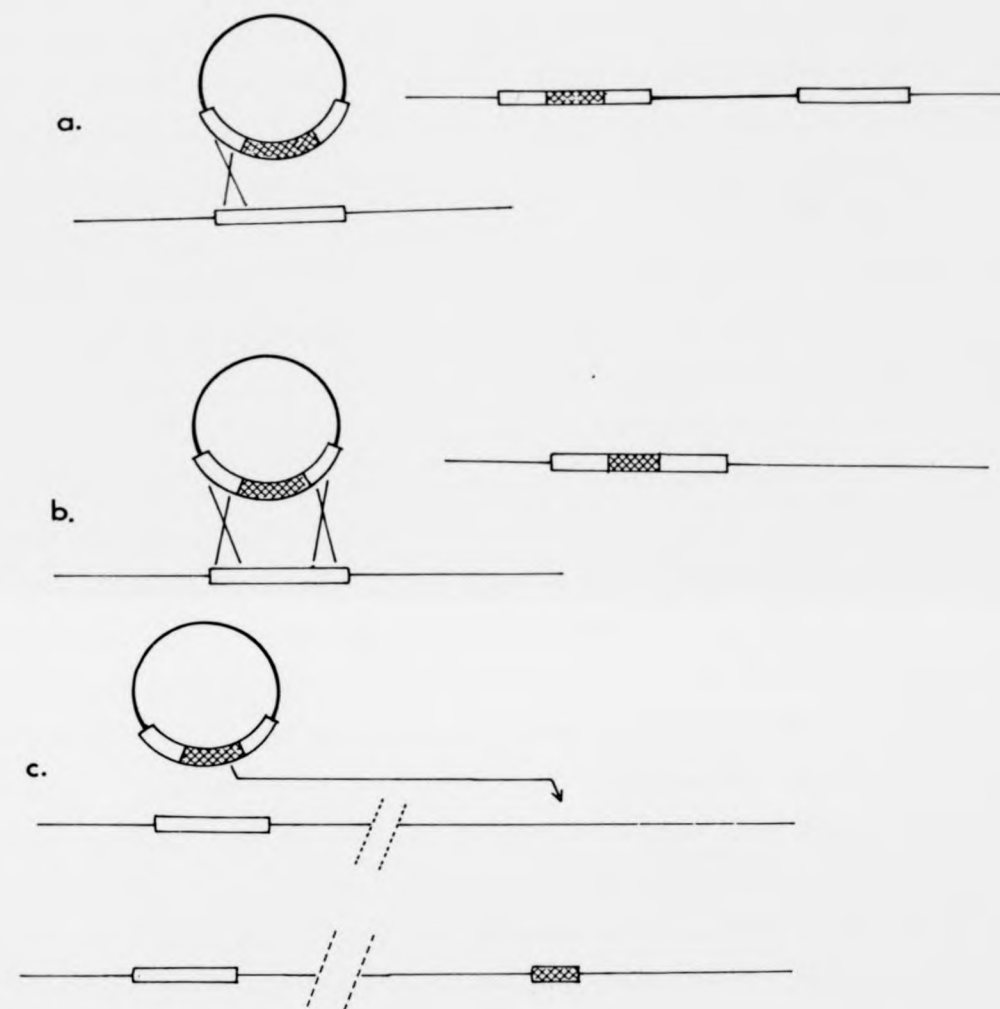
Possible outcomes of the introduction by transduction of *M. xanthus* DNA containing *In5* sequences:

The cloned *M. xanthus* DNA and its homologue in the chromosome is shown as a plain area.

The transposon *In5* is shown as a cross-hatched area

- a. A single recombination event between plasmid and chromosome results in the integration of the entire plasmid.
- b. A double recombination results in the integration of *In5* without the accompanying plasmid. The site of integration is homologous to the site formerly occupied within the plasmid.
- c. The *In5* transposes and becomes integrated in the chromosome without homologous recombination taking place. The site of insertion will be at another point on the chromosome.

Fig. 6-1





(Fig. 6-1). In the first case, there is a single recombination event which brings about the integration of the entire plasmid into the chromosome. An intact copy of the gene remains. In the second instance, two recombinations occur, one on either side of the transposon. This leads to a mutant derived from the wild type, containing a transposon at a known location. The third possibility is that a transposition occurs and the plasmid fails to undergo homologous recombination and is degraded. In this instance, the *In5* will appear at a random location in the chromosome. This is unlikely to be near to the region of the gene fusion. In order to distinguish the three possible outcomes among the transductants, further screening is required. The plasmid insertions, resulting from a single recombination, can be readily distinguished if the plasmid contains a selectable marker in addition to the marker on the transposon. If there is no such marker, the presence or absence of plasmid sequences can be determined by colony hybridisation. The absence of plasmid sequences in a transductant is a strong indication that a gene replacement has been formed through double recombination. This does not exclude the possibility that a random transposition event has occurred instead. It is, therefore, necessary to confirm, by Southern analysis, that the transposon is in the predicted location.

Fig. 6-2

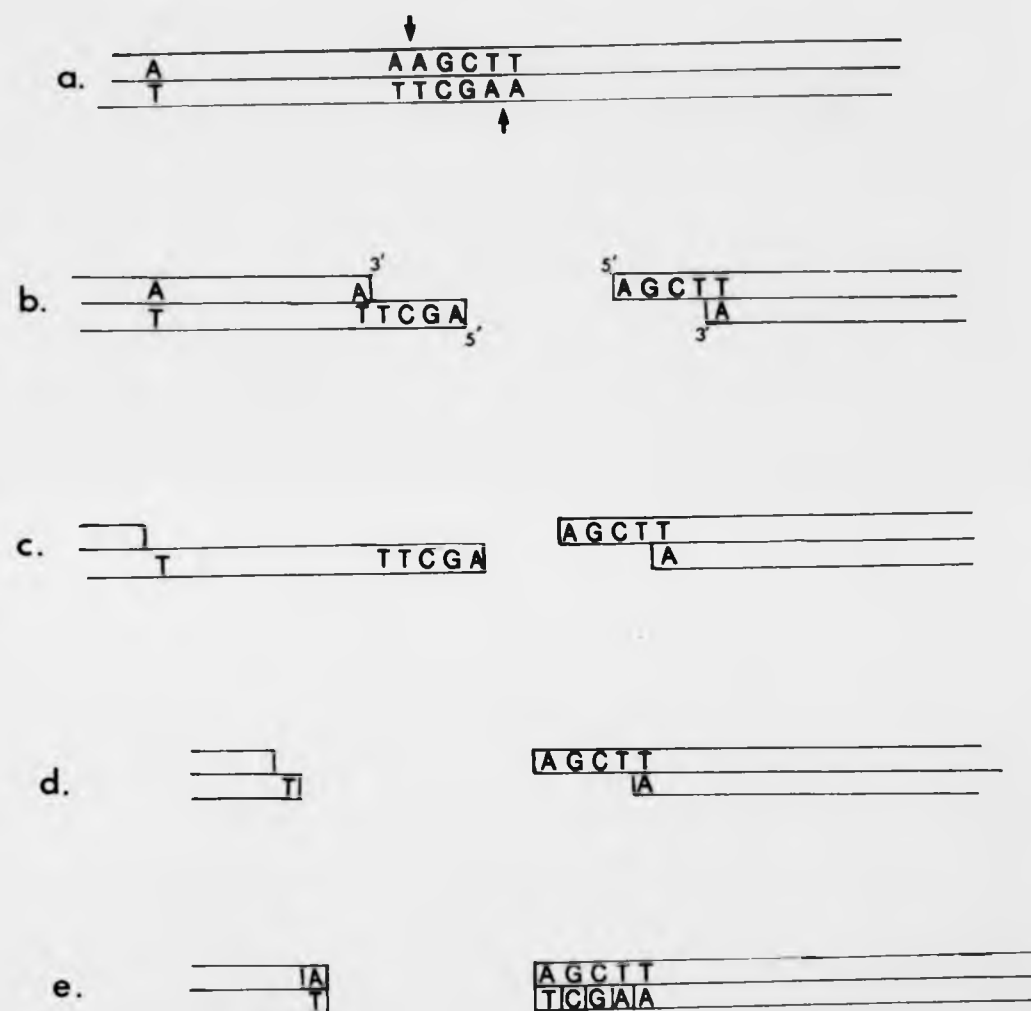
Method for obtaining deletions in the kanamycin resistance gene

The sequence of reactions is shown which was successful in obtaining an internal deletion of the kanamycin resistance gene of PUWMS'.

The HindIII site was preserved. A possible explanation for this is that only one of the cut ends of the DNA was affected. Such a situation is shown here.

- The HindIII site with an AT pair a short distance away
- The enzyme digests to leave a 5' overhang.
- Exonuclease III is used to digest the strand with the 5' overhang. In this instance, one 5' overhang remains undigested.
- S1 nuclease is used to digest single stranded regions. It cannot cleave the last remaining unpaired nucleotide. In this instance, only one of the two single stranded regions is digested.
- T4 DNA polymerase is used to produce blunt ended DNA from any remaining single stranded regions. When this is ligated, a HindIII site is formed.

Fig. 6-2



## 6-3 Preparation of targets for mutagenesis

The plasmids chosen for mutagenesis were pUWM4' and pUWM5' (Figs. 5-1-2 and 5-2-2). These contain <sup>Part or all of</sup> the isgA1 and isgB2 genes respectively. Both of these genes show a considerable increase in expression during sporulation. Both plasmids, therefore, are likely to contain genes which are of importance during sporulation. In the case of pUWM4', sequences have been cloned additional to those cloned in the gene fusion pUWM4. The additional sequences extend downstream from the site in the wild type gene which corresponds to the fusion point in pUWM4. It is possible that pUWM4' includes the entire isgA1 gene. A similar situation was initially believed to be the case with pUWM5', which was derived from pUWM5. Only later, during the course of these mutagenesis experiments, was pUWM5' found to be derived from an internal deletion of plasmid pUWM5. The plasmid did not contain any additional M. xanthus sequences.

The plasmids pUWM4' and pUWM5' both contain a functional kanamycin resistance gene from the promoter-probe vector (originally derived from Tn903) (Oka A. et al. 1981). As kanamycin has to be used to select for Tn5 integration in E. coli, the kanamycin resistance gene in the plasmid had first to be inactivated.

In the instance of pUWM5', there was an ampicillin resistance gene in the plasmid, which could be used as a marker following inactivation of the kanamycin resistance gene. The first attempt to destroy the gene function involved cutting the plasmid at its unique HindIII site which lies within the kanamycin resistance gene. The 5' staggered ends

produced by this enzyme were filled using the DNA polymerase reaction catalysed by the Klenow fragment of E. coli DNA polymerase I. The molecule was recircularised by blunt end ligation. The ligation products were used to transform E. coli to ampicillin resistance. Transformants were tested for kanamycin sensitivity. However, the kanamycin sensitive transductants which were obtained, all showed high levels of spontaneous reversion to kanamycin resistance, presumably due to frameshift mutations which restore the correct reading frame of the kanamycin resistance gene. Such reversion would interfere with selection for Tn5 insertion on the plasmid. This strategy for gene inactivation was not pursued further.

The next attempt to inactivate the kanamycin resistance gene, was to construct plasmids containing large deletions in the gene (Fig. 6-2). Such deletions would not be able to revert. The plasmid was digested with HindIII and then digested using Exonuclease III which progressively digests the 3' strand at the cut end of the DNA. This left a long, single stranded region which, in turn, was removed by digestion with S1 nuclease. Digestion with S1 nuclease, although removing most single stranded DNA, tends to leave behind short 3' or 5' staggered ends. In order to produce blunt ended DNA for ligation, the DNA polymerase reaction was used to fill in the missing nucleotides. This was achieved using T4 DNA polymerase in the presence of all four nucleotides. The plasmid was then recircularised by ligation, and used to transform E. coli to ampicillin resistance. Restriction analysis of the DNA prepared from the transformants, revealed many plasmids derived from pUWMS' but reduced in size to only a few kilobases and lacking most of the M. xanthus DNA. However, one

transformant was found (pUWM5'D23) whose deletion around the HindIII site was too small to be apparent on the gel, but whose phenotype did not revert to kanamycin resistance. A HindIII site was present in this plasmid and had presumably arisen at the point of the deletion.

A different approach had to be made in order to remove kanamycin resistance in pUWM4'. The procedure is shown in Fig. 6-3. The plasmid has no alternative antibiotic resistance markers for selection, once kanamycin resistance is destroyed. Consequently, the gene had to be inactivated by the insertion of another antibiotic resistance gene. This has the effect of exchanging one type of resistance for another. The gene used was one conferring tetracycline resistance derived originally from Tn10. The gene was obtained from the plasmid ColE1::Tn5-132 (Berg D.E. *et al.* 1981). The gene was cut out as a HindIII fragment and eluted from a gel. The fragment was ligated to the plasmid pUWM4', which had previously been linearised using HindIII. A transformant was obtained which was kanamycin sensitive and tetracycline resistant. Restriction analysis revealed the orientation of the inserted sequence. The construct was named pUWM4'tc.

#### 6-4 Mutagenesis of plasmids pUWM4'tc and pUWM5'D23 in E. coli

Mutagenesis of plasmids, using Tn5, was carried out by transduction with a  $\lambda$  phage containing the transposon. The method is described in Materials and Methods (2-7).

Transductants were selected for growth on high concentrations

Fig. 6-3

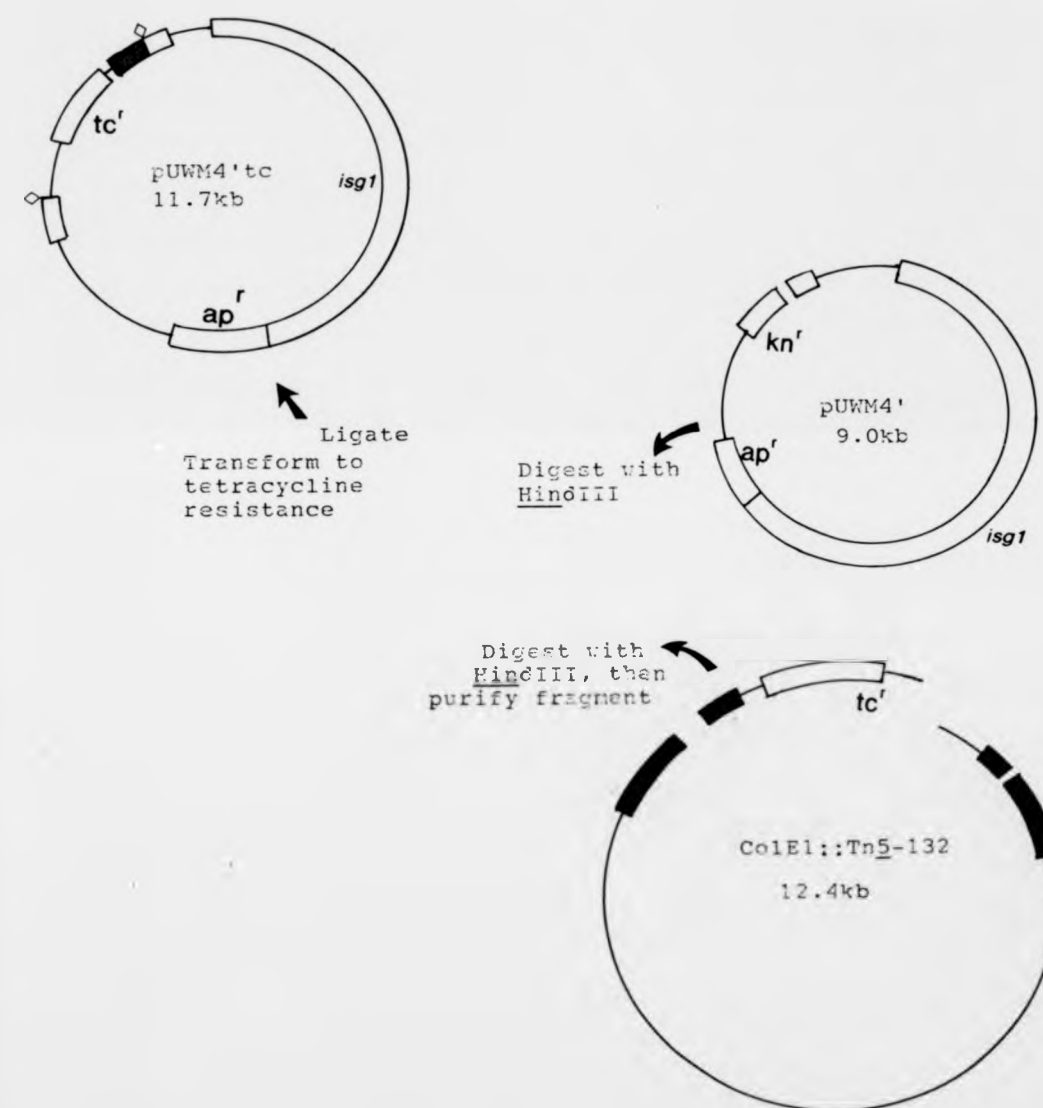
Method for replacing plasmid borne kanamycin resistance with tetracycline resistance

A *Hind*III fragment containing a gene for tetracycline resistance is inserted into the unique *Hind*III site of pUWM4'. This inactivates the gene for kanamycin resistance while simultaneously enabling the plasmid to confer tetracycline resistance.

Sequences from transposon Tn5 are shown darkly shaded.

*Hind*III sites in the resulting plasmid construct are shown as diamond symbols.

Fig 6-3



of kanamycin (300µg/ml). In this way, it was hoped to select for transposon insertions on the plasmid, as opposed to transposon insertions on the chromosome. Multiple gene dosage of the kanamycin resistance gene allows the cell to grow under higher concentrations of kanamycin than would be possible with only a single copy. However, when plasmid DNA was prepared from the small number of transductants obtained, and restriction analysis carried out, only a small proportion of the plasmids were shown to contain a Tn5 insert. (The results are shown in Table 6-1 experiments 1 and 2.) The other transductants had presumably acquired an insert in the chromosome.

In order to avoid time-consuming screening of hundreds of transductants, attempts were made to improve the selection for inserts in the plasmid. One approach was to grow small patches of each transductant strain on a LB plate + kanamycin and then combine ten for each small scale plasmid preparation. Each pooled preparation was then used to transform E. coli to kanamycin resistance. As only plasmid DNA can transform E. coli with any efficiency, the only transformant colonies are those containing a plasmid with a Tn5 insert.

This method was much more rapid as it was not necessary to screen every plasmid for Tn5 inserts by restriction analysis.

However, even the improved method for obtaining transposon inserts was time consuming. Indeed, the frequency of plasmid inserts among the transductants, corresponded to the proportion of DNA in the multicopy plasmid compared to DNA in the chromosome. This suggested that selection for plasmid insertion, using high levels of kanamycin, was not working. It was suspected that high levels of selection in a single step



Table 6-1

## Tn5 mutagenesis of PUWMS-023

	number of transductants tested:	transductants with Tn5 in the plasmid:	transductants with Tn5 in the insert:
1. Selection on Kn <sup>r</sup> °°	85	9	0
2. Selection on Kn <sup>r</sup> °° (transductants tested in batches)	164	5	0
3. Selection on Kn <sup>r</sup> °°; DNA from pooled transductants used in transformation.	600	11	4

Table 6-2

## Tn5 mutagenesis of PUWMS-10

	Transductants resistant to Kn <sup>r</sup> °°:	Total Transductants with Tn5 in plasmid:	Transductants with Tn5 in insert:
1. Transductants selected on Kn <sup>r</sup> °° then picked onto Kn <sup>r</sup> °°	59	49	5
2. Transductants plated onto Kn <sup>r</sup> °° then overlaid to Kn <sup>r</sup> °°	103	96	12



could not select for plasmid borne kanamycin resistance. The small numbers of transductants obtained, may have contained "promoter up" mutations, allowing greater than normal resistance to kanamycin. For this reason, transductions were carried out with selection at the more usual concentration of 50µg/ml. Needless to say, transductants were obtained at far greater frequency than before. The transductant colonies were then picked onto LB plates containing kanamycin at 300µg/ml. A small proportion of transductants grew vigorously under these conditions. The remainder grew only slowly. The vigorously growing colonies were picked for DNA preparation and restriction mapping. Nearly all were found to contain Tn5 inserts in the plasmid. A slightly different approach was tried, where the two step selection was carried out on a single plate. Here, the transductants were plated out on LB containing kanamycin at 50µg/ml. The plates were incubated for 8 hours. At this stage, minute, barely visible colonies had appeared in large numbers. The concentration of kanamycin in the agar was then raised to 300µg/ml, by overlaying with LB soft agar containing the kanamycin. The plates were incubated overnight. Again, nearly all the transductants tested were found to contain an insert in the plasmid (Table 6-2).

#### 6-5 Mapping the positions of Tn5 inserts

The sequence of the transposon Tn5 is known, and so the position of any restriction site can be predicted. The transposon consists of a central, unique region carrying antibiotic resistance genes. This is surrounded by a pair of

ISSO sequences which are inverted repeats. Their restriction sites are therefore mirror images of one another.

Restriction analysis was carried out upon the plasmids containing Tn5 inserts. Restriction digests were carried out using a variety of enzymes, known to cut both in the transposon and in the surrounding plasmid. Certain enzymes cut within the ISSO regions: BglII, HindIII, PstI, PvuII and XhoI. These could be used to map the position of a transposon insert without regard to the orientation of the transposon.

Determining the orientation of the transposon was more of a problem. It was necessary to use an enzyme which cut the transposon only within the unique region, for example, BamHI, SalI or SmaI. In the case of PUWM4'tc, sites cut by SmaI were too numerous to be mapped in the plasmid, and could not, as a consequence, be used to map Tn5 inserts. Furthermore, the three restriction sites used were close to the centre of the transposon. Therefore, differences in orientation were not always apparent as differences in fragment size. However, it was not always considered necessary to determine the orientation of an inserted transposon. The main reason for mapping Tn5 inserts was to investigate any mutagenic effect. This is unlikely to be affected by orientation.

The positions of the Tn5 sequences in PUWM4'tc are shown in Fig. 6-4 and those in PUWM5'D23 are shown in Fig. 6-5.

6-6 Preparation of Tn5 inserts in M. xanthus using gene replacement by plasmid borne Tn5 inserts.

Plasmids, with transposon inserts of interest, were

Fig. 6-4

## Tn5 inserts in pUWM4'tc

This shows plasmid pUWM4'tc with the *M. xanthus* DNA shown as a linear map. This linear map is the same as that shown in Fig. 5-1-2. The kanamycin resistance gene is shown inactivated by the insertion of a fragment containing the tetracycline resistance gene.

The positions of inserts obtained in the *M. xanthus* DNA are shown. Those used in subsequent experiments are shown as open triangles and are described in Table 6-3. Other inserts were obtained which were not used subsequently. These are shown as open diamonds.

Fig. 6-4

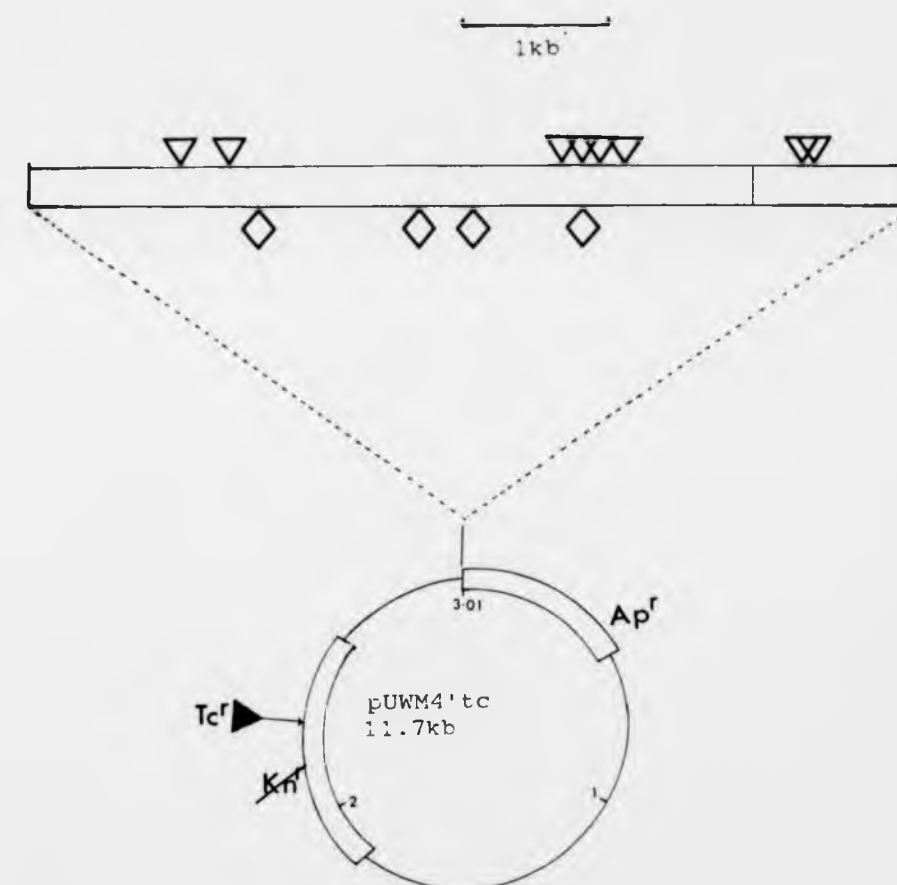


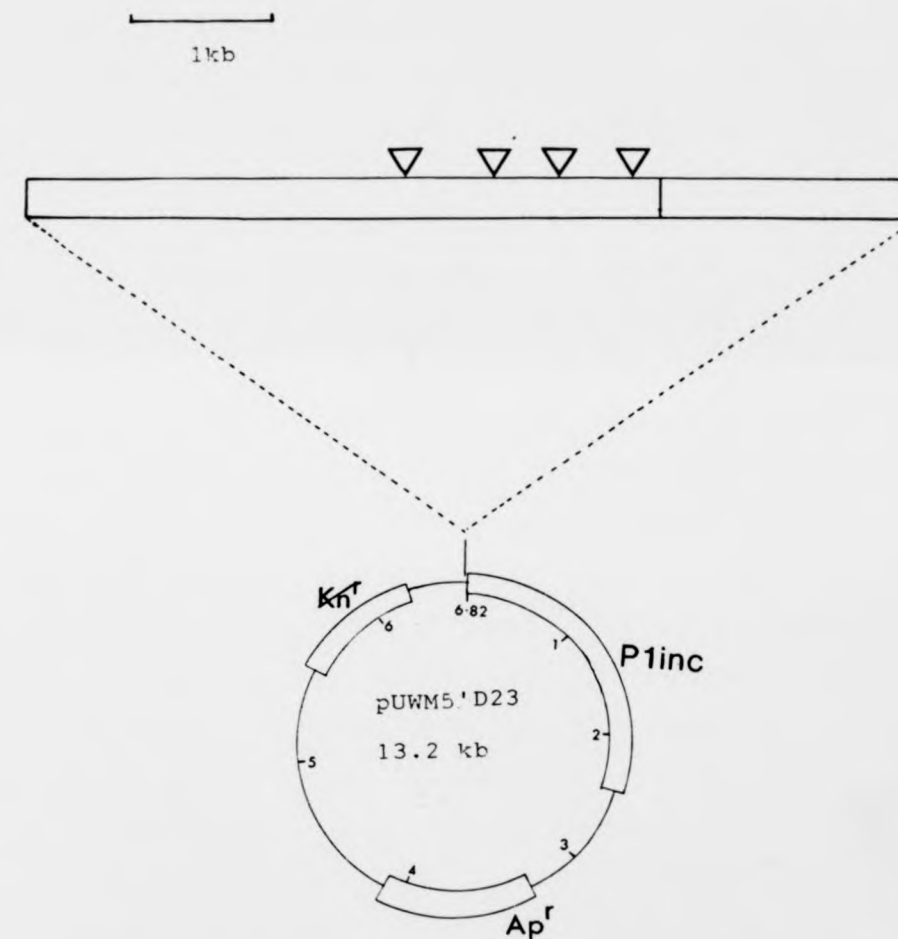
Fig. 6-5

Tn5 inserts in pUWM5'D23

This shows plasmid pUWM5'D23 with the *M. xanthus* DNA shown as a linear map. This linear map is the same as that shown in Fig. 5-2-2. The kanamycin resistance gene is shown as inactivated.

The positions of inserts obtained in the *M. xanthus* DNA are shown as open triangles and are described in Table 6-3.

Fig. 6-5



packaged in P1 phage and used to transduce M. xanthus strain DK1622 to kanamycin resistance. The three possible outcomes of such a transduction are shown in Fig. 6-1. In order to distinguish a gene replacement event from the integration of the entire plasmid, it was necessary to screen for the presence or absence of the plasmid sequences. In the case of pUWM4' derivatives, the plasmid contained a functional gene for tetracycline resistance. Transductants were picked onto oxytetracycline plates and kanamycin plates. Oxytetracycline is preferred to tetracycline as it has a greater window for selection in M. xanthus. Colonies which had acquired oxytetracycline resistance, in addition to kanamycin resistance, were assumed to contain an entire integrated plasmid. However, a minority of the transductants (Table 6-3) were resistant only to kanamycin. These were possible gene replacements and were retained for further analysis.

Transductants from Tn5 inserts in pUWM5'D23 could not be screened in the same way; there was no tetracycline resistance gene on the plasmid. The plasmid contained a gene for ampicillin resistance. Although M. xanthus is naturally resistant to ampicillin, it is reported that the gene for ampicillin resistance can confer cephalosporin resistance in M. xanthus (O'Connor K. and Zusman D.R. 1986). However, when DK1622 was tested on DCY medium with 100µg/ml of cephalosporin, the cells grew regardless. In view of this high level of natural resistance, it was decided to screen for the presence of integrated plasmid by hybridisation to radioactively labelled plasmid. Transductants were picked onto plates, and allowed to grow for 2-3 days. The colonies were transferred to nitrocellulose, then lysed in situ and the DNA bound to the

nitrocellulose (see Materials and Methods 2-28). The nitrocellulose was hybridised using the plasmid pDAH122, which had been radioactively labelled. The areas to which labelled plasmid had bound were visualised by autoradiography. There was a clear distinction between colonies containing integrated plasmid, which were represented by dark spots, and the smaller number of colonies without integrated plasmid, which were represented as gaps in the array of dark spots. It was these colonies, without integrated plasmid, which were possibly the result of gene replacements. These results are summarised in Table 6-4.

It was then necessary to establish that transposon Tn5 had become inserted in the region homologous to the point of insertion in the plasmid. A number of colonies identified as lacking integrated plasmid, were grown as broth culture. Chromosomal DNA was prepared from these. The DNA was digested, using SalI, and run on a 0.6% agarose gel. The DNA on the gel was blotted onto nitrocellulose. The nitrocellulose was then hybridised using pUWM4' or pUWM5' as appropriate. Under these circumstances, the region of the M. xanthus chromosome homologous to the probe (if uninterrupted) should be revealed as a single band, as neither sequence contains any SalI sites. However, Tn5 contains a SalI site. Its presence will be revealed as two bands instead of one, and its position may be deduced from the sizes of the two bands. The band sizes revealed on Southern blots of the mutants, derived from pUWM5' insertion strains, are shown in Table 6-6. Some of the strains indeed contained a Tn5 insert at the predicted location. Others contained no insert within the region of homology. The results are summarised in Table 6-4.

Table 6-3

Mutagenesis of DK1622 using PUWM4<sup>+</sup>tc  
containing Tn5 inserts

201

Strain:	no. of transductants kn, tc <sup>r</sup> / total transductants tested:	no. of gene replacements confirmed by Southern analysis / total transductants tested by Southern analysis:
PUWM4 <sup>+</sup> ::Tn5(-4000)	0/74	-
PUWM4 <sup>+</sup> ::Tn5(-3600)	1/69	0/1
PUWM4 <sup>+</sup> ::Tn5(-1300)	2/25	0/1
PUWM4 <sup>+</sup> ::Tn5(-1100)	4/18	-
PUWM4 <sup>+</sup> ::Tn5(-1050)	1/25	0/1
PUWM4 <sup>+</sup> ::Tn5(-950)	3/50	-
PUWM4 <sup>+</sup> ::Tn5(+400)	6/100	0/1
PUWM4 <sup>+</sup> ::Tn5(+600)	0/68	-

Table 6-4

Mutagenesis of DK1622 using PUWM5<sup>+</sup>023  
containing Tn5 inserts

Strain:	no. of transductants lacking plasmid sequences / total transductants tested:	no. of gene replacements confirmed by Southern analysis analysis / total transductants tested by Southern analysis:
PUWM5 <sup>+</sup> ::Tn5(-200)	8/300	3/8
PUWM5 <sup>+</sup> ::Tn5(-800)	2/50	3/3
PUWM5 <sup>+</sup> ::Tn5(-1300)	10/45	2/3
PUWM5 <sup>+</sup> ::Tn5(-2000)	3/34	1/1

Table 6-5

Mutagenesis of DK306 using PUWM5<sup>+</sup>023  
containing Tn5 inserts

Strain:	no. of transductants lacking plasmid sequences / total transductants tested:	no. of gene replacements confirmed by Southern analysis analysis / total transductants tested by Southern analysis:
PUWM5 <sup>+</sup> ::Tn5(-200)	N.A.	N.A.
PUWM5 <sup>+</sup> ::Tn5(-800)	5/50	0/0
PUWM5 <sup>+</sup> ::Tn5(-1300)	3/50	0/0
PUWM5 <sup>+</sup> ::Tn5(-2000)	11/50	0/0

N.A. = not attempted



Table 6-6

Southern analysis of strains carrying possible Tn5 inserts

The probe used was pUWM5'. The sizes of the bands are shown to the nearest 0.1kb.

Strain:

OK1622[pUWM5': :Tn5(-2000)]

Transductant:

1.  
9.7  
5.7

OK1622[pUWM5': :Tn5(-1300)]

Transductant:

1.	2.	3.
10.8	10.8	9.1
4.9	4.9	

OK1622[pUWM5': :Tn5(-800)]

Transductant:

1.	2.	3.
12.1	12.1	12.1
3.9	3.9	3.9

OK1622[pUWM5': :Tn5(-200)]

Transductant:

1.	2.	3.	4.	5.	6.
9.1	11.2	11.2	11.2	9.1	9.1

7.	8.
9.1	9.1

OK1622

9.1



The mutants derived from pUWM4' were harder to interpret from Southern analysis (Table 6-7). However, the positions of bands on the Southern would indicate that gene replacement had not occurred. Firstly, the band sizes were not the expected sizes. The sum of the band sizes should be the same for every strain containing Tn5 in the UWM4 region. The sum of the sizes should also be equal to the band size in an unmutated strain, plus 5.8kb (the length of Tn5). The band sizes should be predictable from the position of the transposon in the plasmid. The bands on the Southern did not show any of these characteristics. Secondly, the intensities of the bands were the same for any one track. This is in spite of the fact that many of the inserts in the plasmid were near to one end of the cloned region. If these gave rise to inserts in the chromosome, the intensities of the two bands would be very different. This was indeed the case when gene replacements were achieved in the lsg82 region. Thirdly, the blot was reprobed using pUWM4D. This is a derivative of plasmid pUWM4 carrying a large internal deletion and is described in Chapter 7 (Fig. 7-2). When this probe was used, exactly the same bands were revealed. If the transductant strains were the result of gene replacement, certain bands would not reappear with this probe. This is because the plasmid pUWM4':Tn5(+400) contains an insert in a region of pUWM4' which is not present in pUWM4D (Fig. 6-4). Any gene replacement strain, derived from either of these plasmids, would show only one of the two pUWM4' specific bands when probed with pUWM4D.

Table 6-7

Southern analysis of strains carrying possible Tn5 inserts

Chromosomal DNA was digested using Sol 1

The probe used was pUWM4'. The sizes of the bands are shown to the nearest 0.1kb or to the nearest 1kb where the gel could not be read with sufficient accuracy.

Strain:

DK1622[pUWM4':Tn5(-3600)]

25  
14.3

DK1622[pUWM4':Tn5(-1300)]

11.0  
8.1

DK1622[pUWM4':Tn5(-1050)]

14.0  
11.8  
11.3

DK1622[pUWM4':Tn5(+400)]

14.4  
10.2

DK1622

13.2

## 6-7 Phenotypic analysis of mutants

Transductants containing inserts in the lsgB2 region were examined for any mutant phenotype, particularly for defects in sporulation. Strains with possible inserts in the lsgA1 region (in spite of the confusing results results of Southern analysis) were also examined for any mutant phenotype. Strains were tested for their ability to form spores in liquid culture following the addition of glycerol. The same strains were also tested for their ability to undergo developmental sporulation when placed under starvation conditions. Cell suspensions were spotted onto TM medium. After 5-7 days, cells were scraped off and examined for the presence of spherical, phase-bright spores. The viability of these spores was also tested as described in Materials and Methods (2-9). In all the possible mutant strains, there were normal fruiting bodies containing viable spores.

In addition, the transposon inserts: PUWMS'[\u039b5-2000], PUWMS'[\u039b5-1300], PUWMS'[\u039b5-800] and PUWMS'[\u039b5-200], were transduced into a nonmotile DK306 background Table (6-5). In Chapter 4 it was described how the gene fusion is activated to a far greater degree during sporulation in a nonmotile background than during sporulation in a wild type background. This suggests that there is a separate mechanism required for sporulation in the absence of motility, to that required in wild-type strains. A Tn5 insert in the lsgB2 region might be expected to have a strong effect upon sporulation in a nonmotile background. However, the results obtained, showed that gene replacements in the lsgB2 region did not prevent

sporulation from taking place, even in a nonmotile background.

#### 6-8 Concluding remarks

A large number of transposon inserts were obtained in plasmids pUWM5'D23 and pUWM4'tc. The inserts lying within the cloned M. xanthus DNA could be used for site directed mutagenesis of M. xanthus. Insertion mutants were successfully obtained within the isg82 region. The position of these inserts was confirmed by Southern analysis. No mutant phenotype was discernible in any of these strains. This was in spite of the fact that the region showed a large increase in transcription during sporulation. There are several possible reasons for this apparent paradox. Firstly, the four transposon insertions may all lie outside of the transcribed region of the gene. Secondly, an insert might lie within the transcribed region of the M. xanthus gene but be downstream of the translational termination signal (Fig. 6-6). However, neither reason is particularly convincing. The four inserts are evenly distributed along the genome (Fig. 6-5). It would be probable that at least one of these would disrupt gene function. A third possibility is that the promoter of the isg82 gene is within 200bp of the fusion point. Under these circumstances, gene replacement would not disrupt the gene (Fig. 6-6). In Chapter 7, evidence is presented that the sporulation induced promoter lies downstream of the insert at -1300bp. Other evidence appears to define the promoter region to a 700bp region downstream of the StuI site (Fig. 5-2-2). A fourth possibility should be considered. Gene function may

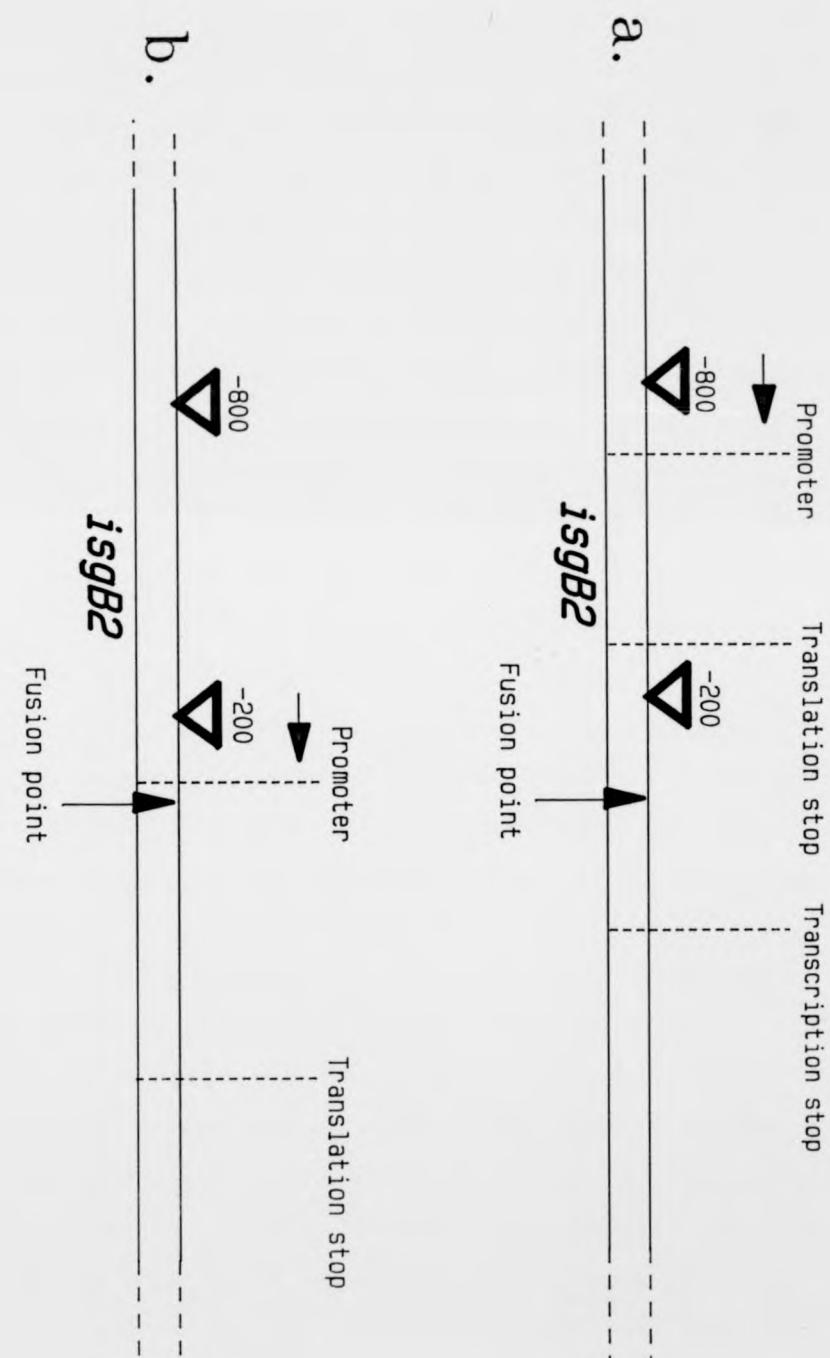
Fig. 6-6

Diagram showing two possible ways in which the *isgB2* gene could escape inactivation by the *Tn5* inserts already obtained (Fig. 6-5).

In a. the promoter and translation stop site both lie between the -800 and -200 inserts. The transcription terminator lies beyond the fusion point, enabling the promoter to direct expression of *lacZ* when the gene exists as a gene fusion.

In b. the promoter lies within 200bp of the fusion point. This would explain the properties of the gene fusion and also account for the lack of a mutant phenotype when *Tn5* was present at -200bp.

Fig. 6-6



have been inactivated by transposon insertion, but without any effect upon sporulation. This was the finding of Kroos L. et al. (1986). Here, a global analysis of gene expression using Tn5 lac insertions, revealed that insertion of the transposon into the transcription unit rarely had any effect upon sporulation. The hypothesis of alternative sporulation pathways was discussed in Chapter 4 (4-7), with particular relevance to the isgB2 region. The absence of motility appears to activate the isgB2 promoter during sporulation. With this in mind, the Tn5 inserts in the UWM5 region were tested in a nonmotile background (This chapter 6-7). Even under these circumstances, viable spores were still able to form. However, the tests used to detect viable spores were purely qualitative. It is conceivable that quantitative comparisons of spore numbers and comparisons of the time taken for sporulation to occur, might reveal differences between mutated strains and the original wild type strain.

Attempts were made to obtain Tn5 insertion mutants in the isgA1 region. In spite of a large number of plasmid based inserts being obtained, no transposon inserts could be obtained from these. Southern analysis of transductants suspected of being gene replacements, revealed patterns of bands which could not be interpreted but which were not gene replacements. Such an outcome may have arisen due to the lack of P1inc sequences in the plasmid. Plasmids without such sequences cannot readily form concatamers with the phage genome. Instead, plasmid multimers are packaged (O'Connor K. and Zusman D.R. 1983). Although gene replacement through recombination can still occur, the plasmid can also integrate at other sites in the chromosome. Separate ISS0 regions, from

different copies of Tn5 in the concatamer, can function together as a giant transposon. A mixture of plasmid and transposon sequences can then transpose to random locations in the chromosome (Downard J. 1988). A further possible complication, would be internal recombination within the plasmid. The tetracycline resistance gene in the plasmid has a 300bp section of an ISSOL region (Fig. 6-3). This is because the fragment was obtained from Tn5::132. The ISSOL region might recombine with its homologue in the Tn5 insert. In some cases, this could lead to the partial loss of the integrated plasmid. This would account for the loss of tetracycline resistance in the transductants tested. Such a phenotype would normally be associated with a gene replacement, resulting from double recombination.

The inability to obtain genuine gene replacements in the isgA1 region may be due to the fact that such a mutation is lethal. Indeed, this inability to obtain gene replacements, contrasts with the ease at which they were obtained for the isgB2 region (Tables 6-3 to 6-5 inclusive). For this reason, it is possible that the isgA1 region is essential to the cell. If so, this would prove the usefulness of the plasmid based promoter-probe. Such a gene fusion, involving an essential gene, could not be isolated as a transposon-based gene fusion. Such a technique is almost certain to inactivate the gene. However, the hypothesis that the isgA1 gene is essential, is still far from proven. It would be necessary to screen a far larger number of transductants for possible gene replacements. Presumably, the entire UWM4 clone is not part of a single, essential operon. Otherwise, the integration of the plasmid would have been a lethal event, and the gene fusion could not have been obtained in the first place.



## CHAPTER 7

Analysis of the functional  
regions of gene fusions by  
means of site-directed  
mutagenesis



## 7-1 Introduction

In the previous chapter, it was described how transposon inserts were obtained in cloned M. xanthus DNA localised to the regions of the gene lsgB2 and possibly also in gene lsgA1. In the case of lsgA1, Southern analysis gave confusing results. However, in neither case was there any discernible phenotypic effect. This chapter describes how site directed mutagenesis was used, not upon wild type M. xanthus, but upon cloned gene fusions, which were then reintroduced into M. xanthus (Table 7-1). In this way, the effect upon transcription of the reporter gene, lacZ, could be observed. The aim of this study was to map the sites required for developmentally regulated transcription. Three forms of site-directed mutagenesis were used. In one of these, the transposon gamma-delta (Tn1000) was used to obtain inserts in plasmids. It is easy to obtain mutants using this transposon: gamma-delta inserts in a plasmid can be obtained by an E. coli x E. coli conjugation. Unlike most forms of transposon mutagenesis, antibiotic selection is not required. Therefore, it was not necessary to inactivate the existing kanamycin resistance gene (as is necessary for Tn5 mutagenesis).

For the second form of site directed mutagenesis, transposon Tn5 was used. In this instance, use was made of the four inserts already obtained in pUWM5' (see Chapter 6). These already contain the original gene fusion together with part of the lacZ gene. A functional lacZ gene can be reconstructed in

these plasmids, and so restore the gene fusion.

The third type of mutagenesis used, was the insertion of the omega fragment in a plasmid containing a promoter fusion. The omega fragment is a cassette gene, custom designed for in vitro mutagenesis.

#### 7-2 Transduction of mutated gene fusions into M. xanthus; the principles involved

The gene fusions described in this chapter were used to transduce M. xanthus to kanamycin resistance. The plasmids selected for this, are listed in Table 7-1. The recipient strain was DK306. This strain had been found to sporulate more efficiently than wild type, upon addition of glycerol. This was probably because its cells, unlike those of wild type strains, show no tendency to agglutinate when grown in liquid culture. It had already been observed that the insides of cell clumps contained large numbers of vegetative cells even after the induction of sporulation by glycerol. A promoter probe plasmid, containing a transposon insert, can recombine into the chromosome in either of two ways (Fig. 7-1). Only one of the possible outcomes leads to a mutation in the gene fused to lacZ. The other leads to a mutation in the copy of the gene already present in the chromosome. In the instance of pUWM5::Tn5(-1300), the only functional kanamycin resistance gene is within the transposon. A third outcome is possible under these circumstances, namely gene replacement through double recombination (Fig. 6-1). However, these double recombinants could be distinguished by picking transductants

Table 7-1

Plasmids used in transduction experiments in this chapter

Plasmid name:	Method of construction:	See text:
PUWM40::Y6(-5000)	<u>gamma-delta</u>	Section 7-3-1 and Fig. 7-3
PUWM40::Y6(-3100)	mutagenesis of	
PUWM40::Y6(-1200)	plasmid PUWM40	
PUWM40::Y6(-200)		
PUWM5::Tn5(-1300)	Gene fusion reconstructed from plasmid PUWM5'::Tn5(-1300)	Section 7-4-1 and Fig. 7-5
PUWM5D::Ω	Insertion of the omega fragment into the <u>StuI</u> site of PUWM5D	section 7-5-1 and Fig. 7-7

Fig. 7-1

Possible outcomes of the transduction of a gene fusion containing a transposon

In a. the transposon is located within the gene fusion.

In b. the transposon is located instead in the formerly intact copy of the gene.

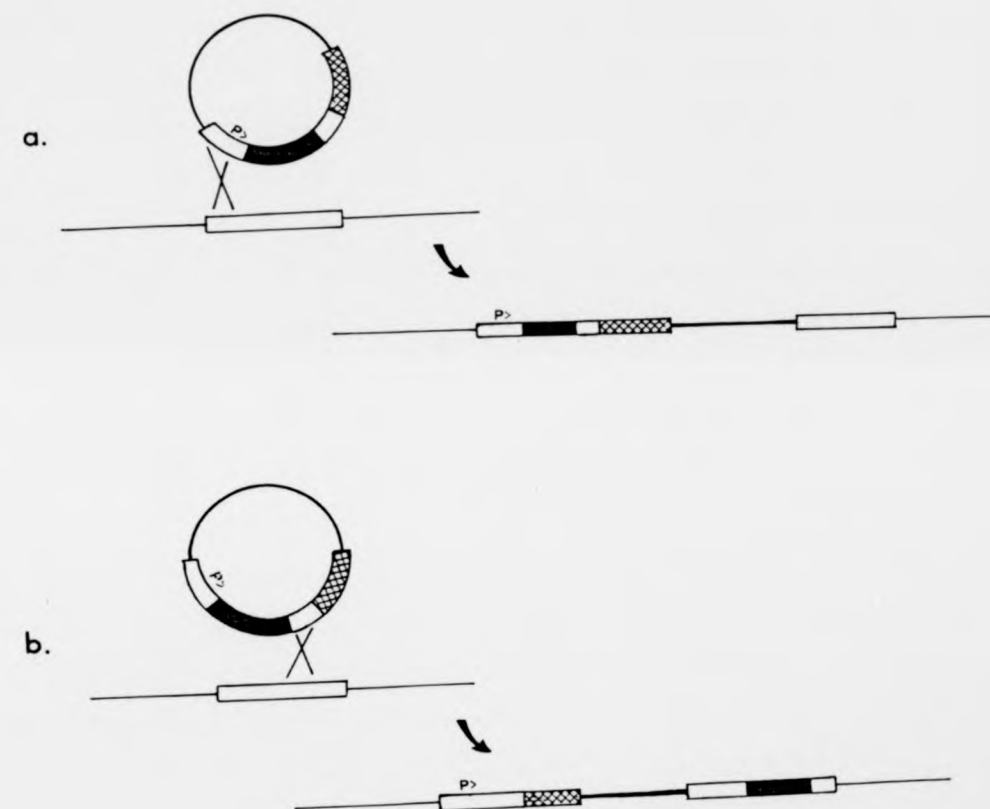
The intact gene and gene fusion to lacZ are both shown as unshaded boxes.

The transposon Tn5 is shown darkly shaded.

A heavy line indicates other plasmid sequences.

A thin line indicates flanking M. xanthus chromosomal sequences.

Fig. 7-1



onto DCY medium containing X-gal. About half of the transductants showed no discernible  $\beta$ -galactosidase expression. It was assumed that these had no lacZ gene and were double recombinants. These would be identical to the gene replacements obtained already, using pUWM5':Tn5(-1300) (Chapter 6). Previous experience of screening large numbers of promoter probe fusions in the M. xanthus genome (Chapter 3), would indicate that, regardless of its position, there is nearly always some detectable expression from a promoterless lacZ gene. Consequently, the blue coloured colonies were assumed to represent both of the possible outcomes of a single recombination event (Fig. 7-1). The transducing phage, obtained by packaging plasmid pUWM5':Tn5(-1300), was also used to transduce LS202. This strain is a sporulation mutant in the csg class. This allowed the activity of the gene fusion lsgB2>lacZ to be tested in this particular class of sporulation mutant.

Several transductants, derived from each of the mutated plasmids, were picked from plates for further analysis. In the case of pUWM5':Tn5(-1300) (described in the previous paragraph), only blue colonies were chosen. Broth cultures of DCY were prepared of each transductant. These were induced to sporulate by adding glycerol. Assays were made for  $\beta$ -galactosidase activity in vegetative cells and spores. The transductants tested this way would be expected to contain both forms of transductant shown in Fig. 7-1. For this reason, Southern analysis was performed upon the same clones. This would allow the two types of transductant to be distinguished. It was anticipated that this would provide an internal control for the experiment. Where the mutation was present in the

fusion gene, this might alter the inducibility of the gene during sporulation. However, if the mutant was in the alternative location (in the chromosomal copy of the gene), the inducibility of the gene fusion would be unaffected.

The findings of these experiments are described in the remainder of this chapter.

### 7-3 Use of transposon gamma-delta

#### 7-3-1 gamma-delta mutagenesis of gene fusions isgA1>lacZ and isgB2>lacZ

This method for mutagenesis, targeted to a plasmid, was described by Guyer A.S. (1983). It is described in Materials and Methods (2-8). The plasmid to be mutagenised is introduced into the conjugation proficient strain RB308. The donor strain is then crossed with a recipient strain, with selection for the plasmid to be mutagenised. The F plasmid is transferred from donor to recipient strain by conjugation. Under these circumstances, the plasmid under mutagenesis can only be cotransferred to the recipient strain if it has formed a cointegrate with the F plasmid. The formation of cointegrates is an essential step in the transposition of gamma-delta. The cointegrate is then resolved leaving the original copy of the transposon unchanged, and a new copy at the site where the cointegrate had been formed. Consequently, plasmids released from the cointegrate should all have acquired a gamma-delta insert.

Plasmids PUWM4 and PUWM5 were conjugated from RB308 to

MC1061. Transconjugant colonies were obtained, and plasmid minipreps were made of these. Restriction analysis was carried out upon the plasmids. It was clear that most consisted of a plasmid plus an insert. However, many of the clones appeared to consist of a multimeric plasmid with one transposon insert. (This multimerisation has previously been observed with plasmids in a recA<sup>+</sup> strain (Berg C.M. et al. 1989).) These would be unsuitable for transcriptional analysis. If transduced into M. xanthus, clones would be produced with several, tandemly repeated gene fusions. Only one gene fusion would be interrupted by a transposon. There were, however, many monomeric plasmids. However, the insert was usually outside of the M. xanthus DNA. Furthermore, even in the few cases where there was an insert in the M. xanthus DNA, it was a long distance from the gene fusion and therefore unlikely to influence transcription.

In order to increase the proportion of usable inserts in the cloned M. xanthus DNA, several changes were made in the procedure. The plasmid was reduced in size by digesting with DraI, then religating (Fig 7-2). This has the effect of removing the lacY and lacA genes, together with the ampicillin resistance gene and P<sub>linc</sub> region. The resulting plasmids were named pUWM4D and pUWM5D. By removing part of the target plasmid, the cloned M. xanthus DNA, a more likely site for gamma-delta insertion. The second change was to add X-gal to the selection plates used to obtain transconjugants. In the initial experiments, it had been found that the only gamma-delta inserts which ever completely eliminated expression of  $\beta$ -galactosidase, were inserts within the lacZ gene. An initial screen, using X-gal, made it possible to



Fig. 7-2

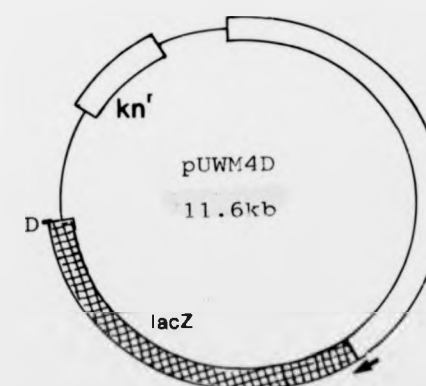
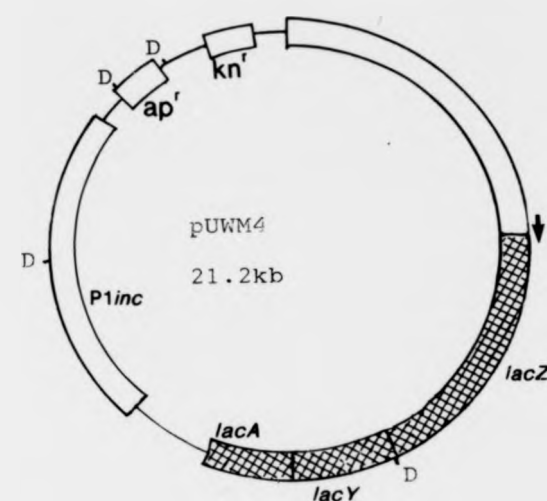
Size reduction of plasmids containing gene fusions

Plasmid pUWM4 is shown with DraI sites marked.

Plasmid pUWM4D is shown. This was produced by digesting pUWM4 with DraI then ligating to produce a greatly reduced plasmid. This plasmid still contains a functional lacZ gene, a plasmid origin and a kanamycin resistance gene, together with the cloned M. xanthus DNA. The direction of transcription in the gene fusion is indicated by an arrow. The lac transcription unit is shown cross-hatched.

Plasmid pUWM5 was reduced in size by the same method.

Fig. 7-2





eliminate inserts within the lacZ gene, from further investigation. Thirdly, a recA<sup>-</sup> strain (HB101 or CSH26) was used as recipient in conjugation. This might be expected to reduce the problem of multimers. However, this would not prevent multimers forming within the donor strain RB308. Using these three changes in the method, additional inserts were obtained (Table 7-1) (Fig. 7-3).

7-3-2 Transduction of M. xanthus using plasmid PUWM4D containing gamma-delta insertions

Four plasmids, containing gamma-delta and derived from PUWM4D, were used to transduce M. xanthus (Table 7-1). Four colonies were selected from each transduction. These 16 resulting strains are listed in Table 7-2. The table also shows the  $\beta$ -galactosidase activity before and after induction of sporulation. Only in one clone, was there any appreciable increase in expression of the gene fusion.

Southern analysis was also carried out upon DNA from all 16 clones (Table 7-3). Chromosomal DNA samples were digested with BscI (an isoschizomer of ClaI). The strain UWM4 containing the gene fusion but no gamma-delta, was run alongside for comparison. The probe used, was PUWM4'. It was clear that the transductants had acquired one and sometimes two extra bands. This was surprising. The enzyme BscI cuts once within gamma-delta. It would therefore be expected that the clones, containing the transposon, would have lost one of the bands produced in UWM4 and gained two new bands. The sum of the lengths of the new bands would equal the length of the missing band, plus the length of gamma-delta. This was

Table 7-2

Transductant *M. xanthus* strains containing gamma-delta:  
the effect upon expression of the gene fusion sgf1

The original strain is shown, followed by the name of  
the plasmid in square brackets. The figure in round  
brackets refers to an individual clone obtained from the  
transduction.

The name of each strain describes the plasmid used in  
transduction. It does not necessarily indicate that the  
strain contains an entire integrated plasmid.

# denotes a strain found by Southern analysis to  
contain gamma-delta sequences

\* denotes a strain found by Southern analysis to  
contain more than one copy of the gene fusion

The units of  $\beta$ -galactosidase activity are expressed  
per milligram of soluble protein. The definition of the  
units is described in Materials and Methods (2-14).

Strain:	$\beta$ -galactosidase expression:	
	vegetative	sporulation
DK306 [PUWM4D::Y6(-5000)](1)	29.8	23.4
DK306 [PUWM4D::Y6(-5000)](2)*	23.4	42.6
DK306 [PUWM4D::Y6(-5000)](3)*	53.2	95.8
DK306 [PUWM4D::Y6(-5000)](4)	19.1	17.0
DK306 [PUWM4D::Y6(-3100)](1)	68.1	76.6
DK306 [PUWM4D::Y6(-3100)](2)	21.3	95.8
DK306 [PUWM4D::Y6(-3100)](3)*	29.8	106.5
DK306 [PUWM4D::Y6(-3100)](4)	27.6	51.2
DK306 [PUWM4D::Y6(-1200)](1)	2.9	506.9
DK306 [PUWM4D::Y6(-1200)](2)	43.0	46.8
DK306 [PUWM4D::Y6(-1200)](3)	112.8	48.9
DK306 [PUWM4D::Y6(-1200)](4)	27.6	44.7
DK306 [PUWM4D::Y6(-200)](1)	19.1	51.1
DK306 [PUWM4D::Y6(-200)](2)*	106.5	140.5
DK306 [PUWM4D::Y6(-200)](3)*	153.3	127.8
DK306 [PUWM4D::Y6(-200)](4)	29.8	48.9
DK306 [PUWM4]	40.4	922.2

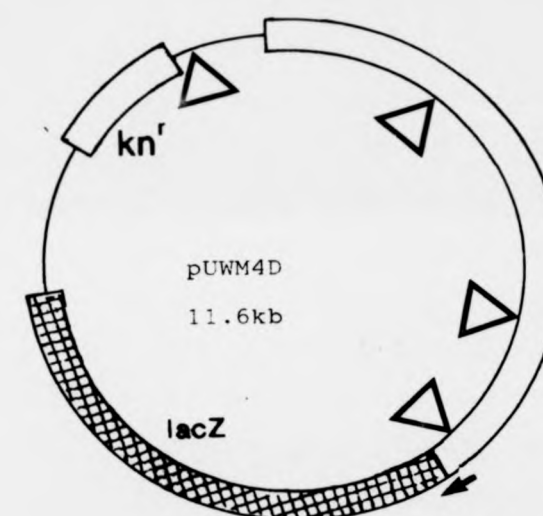
Fig. 7-3

Positions of gamma-delta inserts obtained in pUWM4D.

Plasmid pUWM4D is shown. The direction of transcription in the gene fusion is indicated by an arrow. The lac transcription unit is shown cross-hatched.

Positions of the inserts obtained of transposon gamma-delta are shown as open triangles

Fig. 7-3



evidently not the case.

In an attempt to clarify the form of plasmid which had integrated, the nitrocellulose was stripped of probe and then reprobed using pUWM4 (Table 7-3). The same bands were revealed as before plus one extra (a fragment from the lacZ region). In order to reveal whether the transposon had in fact become integrated, the nitrocellulose was again stripped of probe. A XhoI digest was performed upon a plasmid containing gamma-delta (obtained from D. Gill). The internal fragment, some 3.3kb in length was gel purified. This was used to probe the nitrocellulose filter. This would be expected to reveal two bands from a chromosomal digest. The result is shown in Table 7-3. In fact, only two clones appear to contain any gamma-delta sequences at all. It is clear that the other clones must have integrated the plasmid pUWM4D, without any gamma-delta insert. The interpretation of the bands is shown in Fig. 7-4. Eleven of the strains can be explained as containing an integrated plasmid, pUWM4D. Three of the strains contain the same bands as would be produced from an integrated plasmid, but with one extra band. These can be explained as integrated dimer of the plasmid. The extra band is the equivalent of a linearised monomeric plasmid. Finally, there are two tracks with bands hybridising to gamma-delta sequences. These represent an integrated plasmid pUWM4D. The plasmid, in turn contains an integrated copy of gamma-delta. The transposon lies about 200 bp upstream of the fusion point.

Does the presence or absence of gamma-delta inserts have any effect upon the regulation of the gene fusion? The two clones containing a gamma-delta insert, show significantly higher levels of vegetative expression than do the remaining

Fig. 7-4

Interpretation of Southern analysis upon *M. xanthus* strains transduced by pUWM4 carrying gamma-delta insertions

a. shows the gamma-delta insert within an integrated plasmid monomer (within the gene fusion).

b. shows an integrated plasmid dimer containing  $\alpha$  gene fusion with no insert

The intact gene and gene fusion to lacZ are both shown as unshaded boxes.

The transposon gamma-delta is shown darkly shaded.

Open diamond symbols represent BscI sites. = ClaI

The arrows below a. indicate the fragments hybridising to a gamma-delta specific probe.

The arrows above each map indicate the fragments hybridising to plasmid pUWM4\* and also to plasmid pUWM4.

Fig. 7-4

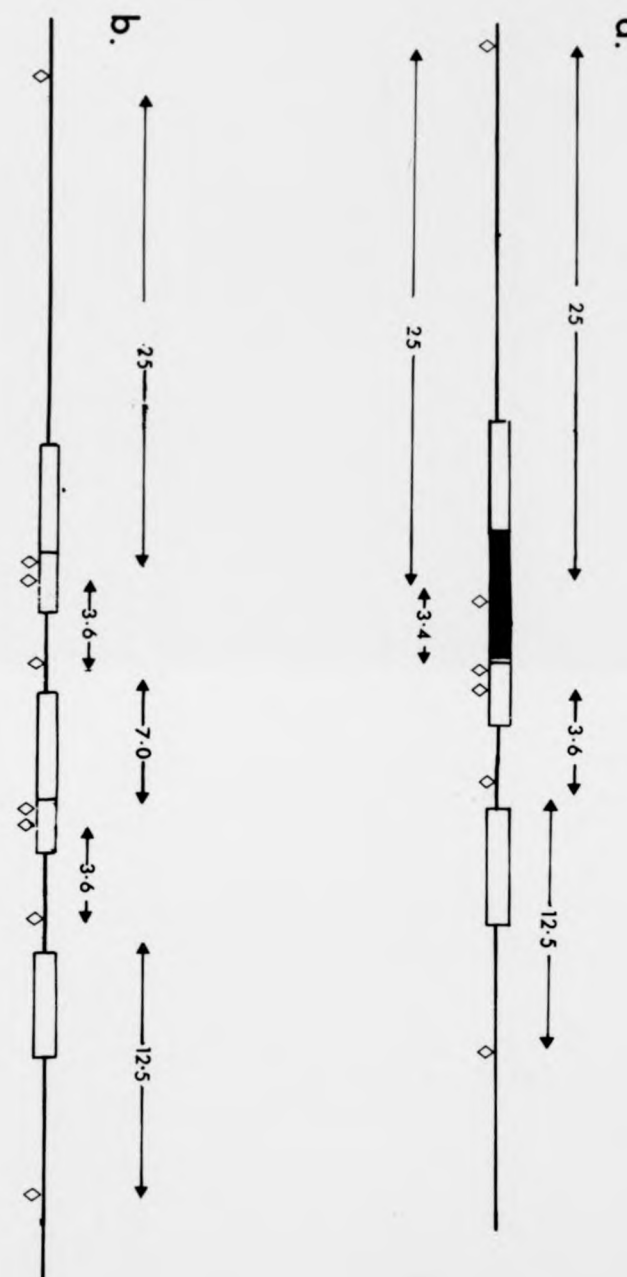


Table 7-3

Southern analysis of strains obtained by transduction of OK306 using pUWM40 derivatives containing gamma-delta

Table 7-3-1

The probe used was pUWM4. The sizes of the bands are shown to the nearest 0.1kb., or to the nearest 1kb where the gel could not be read with sufficient accuracy.

Plasmid used in transduction: pUWM40::Y6(-5000)

Transductant:	(1)	(2)	(3)	(4)
	24	24	24	24
	12.6	12.6	12.6	12.6
		7.0	7.0	
	3.6	3.6	3.6	3.6
	1.8	1.8	1.8	1.8

Plasmid used in transduction: pUWM40::Y6(-3100)

Transductant:	(1)	(2)	(3)	(4)
	24	24	24	24
	12.6	12.6	12.6	12.6
			7.0	
	3.6	3.6	3.6	3.6
	1.8	1.8	1.8	1.8

Plasmid used in transduction: pUWM40::Y6(-1200)

Transductant:	(1)	(2)	(3)	(4)
	24	24	24	24
	12.6	12.6	12.6	12.6
	3.6	3.6	3.6	3.6
	1.8	1.8	1.8	1.8

Plasmid used in transduction: pUWM40::Y6(-200)

Transductant:	(1)	(2)	(3)	(4)
	24	24	24	24
	12.6	12.6	12.6	12.6
	3.6	3.6	3.6	3.6
	1.8	1.8	1.8	1.8

Plasmid used in transduction: pUWM4

24
12.6*
1.8

\* assumed to be a double band

Table 7-3-2

The Southern blot described in table 7-3-1 was rehybridised with a probe specific to gamma-delta. The tracks with hybridising bands are shown. no other tracks contained any hybridising bands.

The sizes of the bands are shown to the nearest 0.1kb., or to the nearest 1kb where the gel could not be read with sufficient accuracy.

Plasmid used in transduction: pUWM40::Y6(-200)

Transductant:

(2)	(3)
24	24
3.4	3.4



14 clones (Table 7-2). Therefore, there is some outward directed promoter activity from gamma-delta. This activity is maintained after induction of sporulation. Indeed, outward reading promoters have previously been identified in gamma-delta within an E. coli host (Lers A. et al. 1989). Of all 16 clones, only one (pUWM4D::Y61200(1)) showed any clear cut increase in expression of the gene fusion. This clone contains a single insert of pUWM4D. Smaller increases in activity were discernible in other clones. The low levels, or total absence, of glycerol induction in 13 clones, containing no gamma-delta, requires an explanation. One possibility is that the conditions for glycerol induction could not be reproduced with every clone. However, all cultures were examined under the phase-contrast microscope, at least 16 hours after the addition of glycerol. All were found to contain spherical spores. Another possibility would be that the integrated plasmid had undergone internal deletions. In this way, some of the M. xanthus sequences, upstream of the gene fusion might have been lost. Such changes would normally be visible as changes in band position in the Southern. Small deletions might, however, go unnoticed. The band representing M. xanthus DNA is extremely large (Table 7-3), and the loss of 1-2kb would go unnoticed. However there is no a priori evidence to suggest that deletions of this kind occur during recombination.

7-4 Mutagenesis of the lsgB2>lacZ gene fusion using a plasmid containing Tn5

7-4-1 Tn5 inserts within pUWMS

As described in Chapter 5, the plasmid pUWMS' was apparently generated by removing an EcoRV fragment from pUWMS. Consequently, it should be possible to reconstruct a gene fusion by adding back the missing fragment. This was attempted using the four Tn5 containing derivatives of UWMS' described in Chapter 6. The plasmids pUWMS':Tn5(-2000), pUWMS':Tn5(-1300), pUWMS':Tn5(-800) and pUWMS':Tn5(-200) were digested with EcoRV, which cuts each plasmid at a single site. The cut ends of the linearised plasmids were then dephosphorylated using E. coli alkaline phosphatase. This enzyme was used in preference to calf intestine alkaline phosphatase, as it can dephosphorylate the blunt ended DNA produced by digestion with EcoRV. The plasmid pDAH122 was digested with EcoRV. This produced two fragments, the smaller of which was cut out as a band on a gel, and extracted by electroelution. Samples of this fragment were then ligated to each of the linearised, dephosphorylated plasmids (Fig. 7-5). The ligation mix was used to transform E. coli to kanamycin resistance. The plates with transformants were sprayed with 10mg/ml 4-MUG in DMSO and examined under UV illumination. This enabled the rapid identification of colonies where a functional lacZ gene had been regenerated. The result could then be confirmed by restriction analysis using EcoRV. A gene fusion was obtained from pUWMS':Tn5(-1300) but not from the

Fig. 7-5

# Construction of pUWM5 derivatives containing Tn5

The reconstruction of Tn5 insertion mutants in pUWM5 from Tn5 insertion mutants already obtained in pUWM5'.

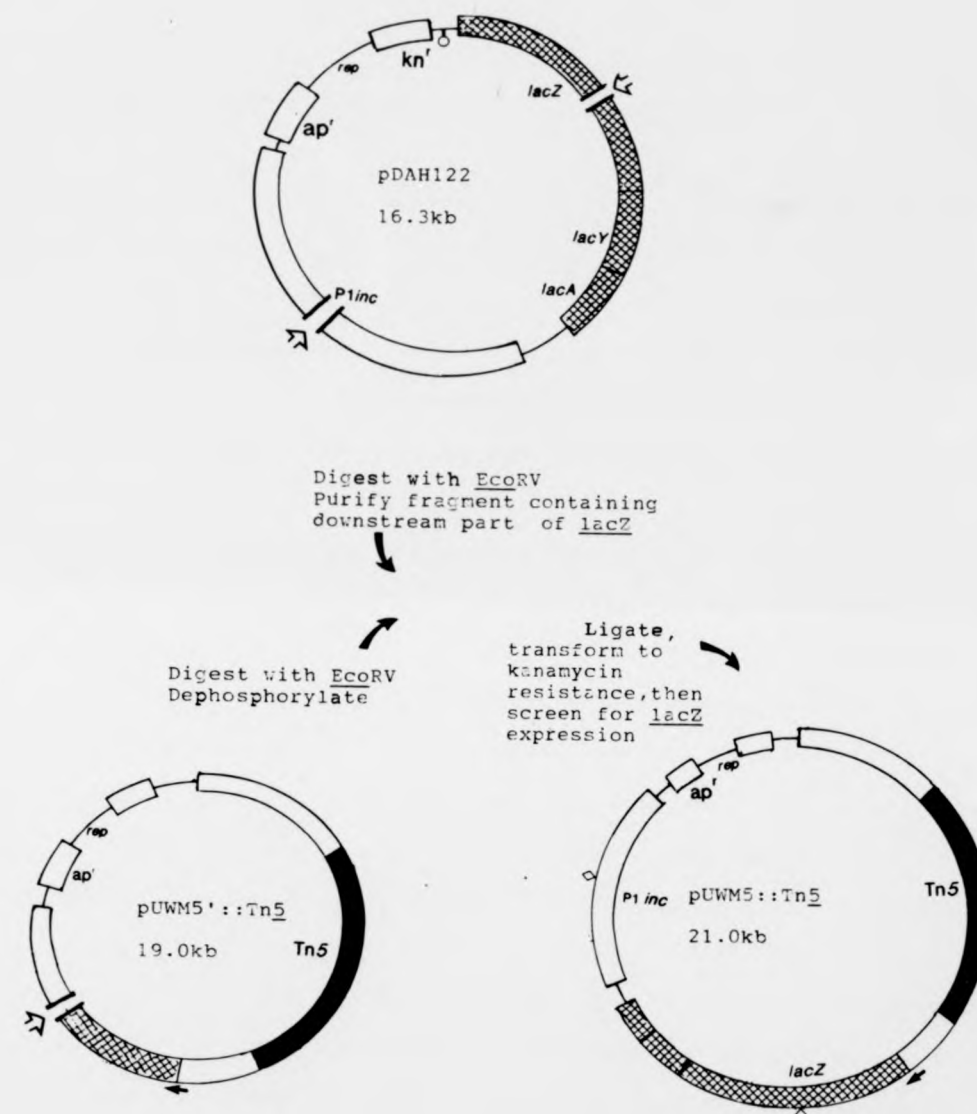
Sequences from transposon Tn5 are shown darkly shaded.

The *lac* transcriptional region is shown cross-hatched.

The direction of transcription into the *lacZ* gene is shown by a small arrow.

*EcoRV* sites in the resulting plasmid construct are shown as diamond symbols.

Fig. 7-5



other three plasmids containing Tn5 inserts.

#### 7-4-2 Transduction of M. xanthus using pUWMS::Tn5(-1300)

An insertion of transposon Tn5 had been obtained in the gene fusion pUWMS. Only one site of insertion had been obtained; at -1300bp relative to the fusion endpoint. Transductants had been picked and screened for  $\beta$ -galactosidase (see section 7-3). Ten transductants expressing  $\beta$ -galactosidase were assayed for  $\beta$ -galactosidase activity both before and after induction of sporulation. Sporulation was induced in liquid culture by the addition of glycerol or DMSO. All clones showed strongly increased expression during sporulation. The results are shown in Table 7-4. Samples of the ten clones were used to prepare chromosomal DNA for Southern analysis. The chromosomal DNA was digested with BamHI and separated on an agarose gel. The Southern blot was probed with pUWMS' and the radioactivity visualised by autoradiography. The probe was then stripped of hybridized probe and hybridised to a different probe, pOAH122. This was specific for plasmid sequences but not for any M. xanthus sequences. In order to confirm the presence of Tn5 sequences, a probe was prepared, specific to the transposon. A plasmid, containing Tn5 was digested with HindIII. This released a 3.4kb fragment, internal to the transposon. The fragment was electroeluted from the gel, then labelled by nick translation. The Southern filter was stripped of the previous probe and then probed with this fragment. The bands produced from the three hybridisation experiments are summarised in Table 7-5.

Table 7-4

$\beta$ -galactosidase expression of strains carrying  
PUWM5::Tn5(-1300)

The units of  $\beta$ -galactosidase activity are expressed per milligram of soluble protein. The definition of the units is described in Materials and Methods (2-14).

The original strain is shown, followed by the name of the plasmid in square brackets. The figure in round brackets refers to an individual clone obtained from the transduction.

An asterisk \* denotes a strain where Southern analysis has shown that the transposon lies within the normally intact copy of the gene and not within the gene fusion.

Strain:	$\beta$ -galactosidase activity:	
	vegetative	sporulation
DK306[PUWM5::Tn5(-1300)](1)	80.9	359
DK306[PUWM5::Tn5(-1300)](2)	83.0	2513
* DK306[PUWM5::Tn5(-1300)](3)	80.7	852
DK306[PUWM5::Tn5(-1300)](4)	93.7	1960
DK306[PUWM5::Tn5(-1300)](5)	83	1176
LS202[PUWM5::Tn5(-1300)](1)	108.6	3109
LS202[PUWM5::Tn5(-1300)](2)	53.2	4984
* LS202[PUWM5::Tn5(-1300)](3)	161.8	1410
LS202[PUWM5::Tn5(-1300)](4)	164.0	4856
LS202[PUWM5::Tn5(-1300)](5)	304.5	4558
DK306[PUWM5]	57.9	4926

Table 7-5

Southern analysis of strains obtained by transduction of DK306 and LS202 using pUWM5::Tn5(-1300)

The probe used was pUWM5'. The same bands were revealed by hybridisation to a probe specific to Tn5. The sizes of the bands are shown to the nearest 0.1 kb., or to the nearest 1kb. where the gel could not be read with sufficient accuracy. An asterisk \* denotes the bands which also hybridised to the plasmid pUWM5.

A BamHI digest chromosomal DNA from strain UWM5 was run on the gel as a control. However, no bands appeared on the Southern blot. This was assumed to be due to poor transfer of the DNA.

DK306[pUWM5::Tn5(-1300)]

Transductant:			
(1)	(2)	(3)	(4)
24*	24*	24*	24*
9.2	9.2		9.2
		4.7	

LS202[pUWM5::Tn5(-1300)]

Transductant:				
(1)	(2)	(3)	(4)	(5)
24*	24*	24*	24*	24*
9.2	9.2		9.2	9.2
		4.7		

The interpretation of these results is shown in Fig. 7-6. Seven of the clones contain a Tn5 insert at a point 1.3kb upstream of the gene fusion. The remaining two contain the Tn5 insert in the intact copy of the lsgB2 gene. Furthermore, neither class of insertion has any effect upon the normal regulation of the gene during sporulation, at least not during chemically induced sporulation. The existence of sporulation regulated expression in the seven clones with an insert in the fused gene suggests that the developmental regulation of the gene fusion in PUWMS, is confined to the region 1.3kb upstream of the site of the fusion. It is just possible, however, that the expression of the fused gene has arisen from copies of the gene fusion which have lost Tn5. This might happen if the homologous regions, on either side of the gene fusion were to recombine (Fig. 6-1). Under these circumstances, the plasmid could excise, then reintegrate in the alternative configuration. The transposon would, instead, lie within the complete copy of the gene, rather than within the gene fusion (Fig. 6-1). However, there is no evidence of this rearrangement occurring on a large scale, as this would be apparent on the Southern. A low level of rearrangement would be a very unlikely reason for the high levels of expression observed during sporulation (Table 7-4).



Fig. 7-6

Interpretation of Southern analysis of strains transduced by Tn5 derivatives of PUWMS

a. shows the Tn5 insert within the gene fusion.

b. shows the Tn5 insert within the formerly intact copy of the gene

The intact gene and gene fusion to *lacZ* are both shown as unshaded boxes.

The transposon Tn5 is shown darkly shaded.

A heavy line indicates other plasmid sequences.

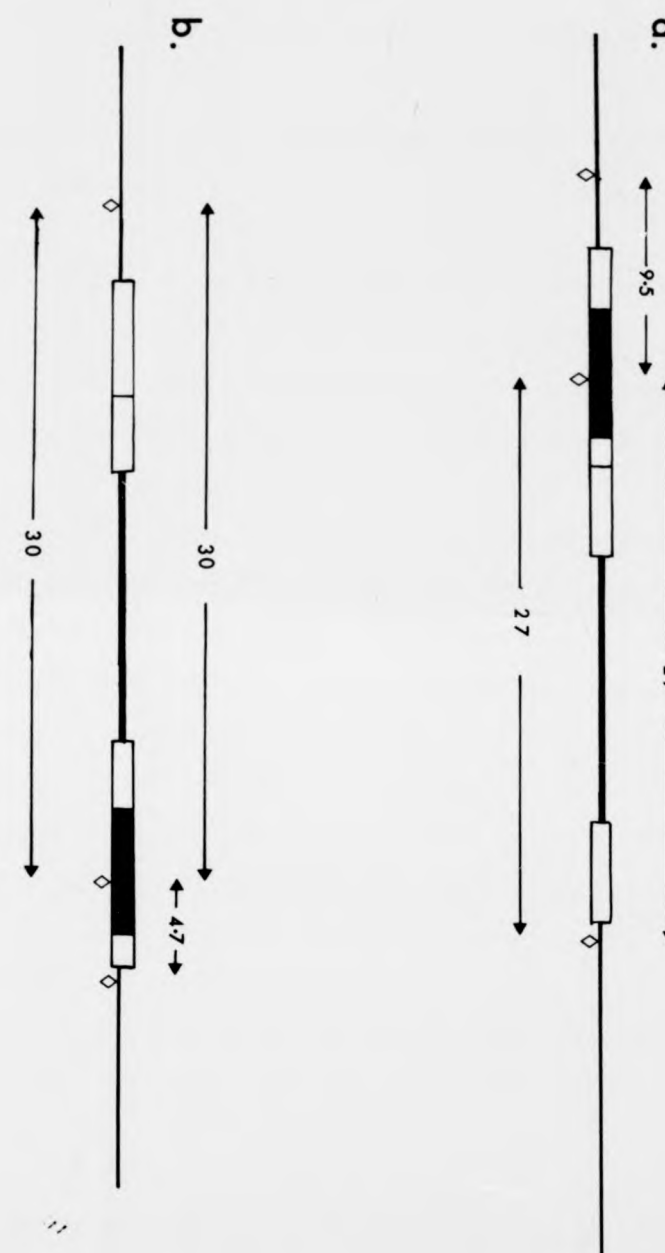
A thin line indicates flanking *M. xanthus* chromosomal sequences.

Open diamond symbols represent BamHI sites.

The arrows below each map indicate the fragments hybridising to plasmid pDAH122.

The arrows above each map indicate the fragments hybridising to plasmid PUWMS' and also to a Tn5 specific probe.

Fig. 7-6.





## 7-5 Mutagenesis of the gene fusion isgB2>lacZ

### 7-5-1 Insertion of the omega fragment upstream of the gene fusion isgB2>lacZ

The omega fragment is a construct designed for in vitro mutagenesis (Prentki P. and Krisch H.M. 1984). It consists of a selectable marker (spectinomycin resistance) flanked by a pair of translation termination signals and a pair of transcription termination signals. (Fig. 7-7). It was obtained as a plasmid PHP45 $\Omega$ , in which the omega fragment is surrounded on either side by a polylinker. The omega fragment was purified on a gel as a SmaI fragment, then electroeluted.

The plasmid used for in vitro mutagenesis was PUWMSD, described in the previous section. This has the advantage over plasmid PUWMS in having a unique StuI site 700bp upstream of the fusion point. (The plasmid PUWMS has a second site near the lacA region). The plasmid PUWMSD was digested with StuI. This enzyme leaves blunt ends when it cuts DNA. These can be ligated to similar blunt ends produced by SmaI. Recombinant plasmids were obtained by selecting for spectinomycin resistance.

### 7-5-2 Transduction of M. xanthus using PUWMSD:: $\Omega$ .

The plasmid PUWMSD:: $\Omega$  was packaged in P1, then used to

Fig. 7-7

Construction of pUWM5D:: $\Omega$ 

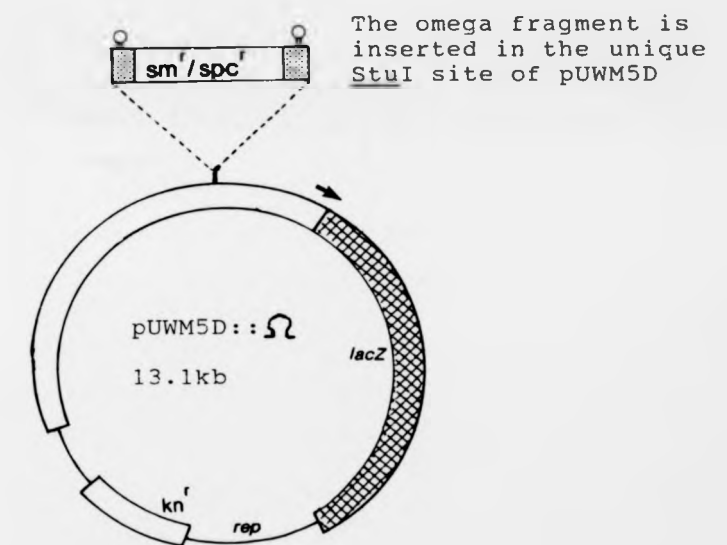
Sequences from the omega fragment (other than the spectinomycin resistance gene) are shown stippled.

The *lac* transcriptional region is shown cross-hatched.

The direction of transcription into the *lacZ* gene is shown by a small arrow.

Transcription terminators within the omega fragment are shown as stalked circles.

Fig. 7-7



transduce *M. xanthus* to kanamycin resistance. As there were two possible outcomes of the plasmid recombining into the genome (Fig 7-1), it was anticipated that the two classes of transductant might express the *lacZ* gene at different levels. However, there were no discernible differences between the transductants, when picked onto X-gal. Four transductants were picked for further analysis. These were grown in liquid culture to log phase, and then induced to sporulate by the addition of glycerol. Samples were taken both before and after induction of sporulation, and assayed for protein and for  $\beta$ -galactosidase. The specific activity of  $\beta$ -galactosidase could then be calculated. The results are shown in Table 7-6. The configuration of the integrated DNA, could not be ascertained from these results. For this reason it was necessary to carry out Southern analysis of chromosomal DNA. Chromosomal DNA was prepared from the four transductant clones and digested with *Bam*HI and *Hind*III combined. A gel was run of these digests and transferred to nitrocellulose. The nitrocellulose was probed using pUWM5'. The results are summarised in Table 7-7. A surprisingly large number of bands was revealed in each track. The filter was then stripped of probe, and reprobed using pDAH122. This was specific for plasmid sequences but not for *M. xanthus* sequences. The pattern of bands revealed was identical. At a later date, when the radioactivity had almost entirely decayed, a different probe was used. A *Bam*HI fragment containing almost the entire omega fragment, was gel purified. This was nick-translated and used to probe the nitrocellulose. The results of this are shown in Table 7-7.

The Southern analysis suggests that there is an

Table 7-6

$\beta$ -galactosidase expression of strains carrying  
PUWM5D:: $\Omega$

The units of  $\beta$ -galactosidase activity are expressed per milligram of soluble protein. The definition of the units is described in Materials and Methods (2-14).

The original strain is shown, followed by the name of the plasmid in square brackets. The figure in round brackets refers to an individual clone obtained from the transduction.

An asterisk \* denotes the strain where Southern analysis has shown that there are no omega sequences within the transduced plasmid.

Strain:	$\beta$ -galactosidase activity:	
	vegetative	sporulation
OK306[PUWM5D:: $\Omega$ ](1)	119.2	1010
OK306[PUWM5D:: $\Omega$ ](2)	129.9	701
OK306[PUWM5D:: $\Omega$ ](3)	240.6	743
* OK306[PUWM5D:: $\Omega$ ](4)	72.4	182.3
OK306[PUWM5]	72.4	1790.3

Table 7-7

Southern analysis of strains obtained by transduction of OK306 using PUWMSD::Ω

The probe used was PUWMS'. The sizes of the bands are shown to the nearest 0.1kb., or to the nearest 1kb where the gel could not be read with sufficient accuracy. An asterisk \* denotes an additional band, which hybridised to a probe specific to the omega fragment. Bands of high intensity are indicated by underlining.

A BamHI/HindIII double digest chromosomal DNA from strain UWMS was run on the gel as a control. However, no bands appeared on the Southern blot. This was assumed to be due to poor transfer of the DNA.

Transductant:

(1)	(2)	(3)	(4)
			12.5
11.6			
	11.2	11.2	<u>11.2</u>
<u>6.6</u>	<u>6.7</u>	<u>6.7</u>	
5.9	5.9	5.9	6.0
<u>4.6</u>	<u>4.6</u>	<u>4.6</u>	
2.0*	2.0*	2.0*	

integrated, multimeric plasmid. The presence of multimers would explain the large number of bands in the Southern analysis. It would also explain the differences between bands in intensity. Autoradiographs of the Southern blot are shown in Fig. 7-8. An interpretation of the Southern analysis is shown in Fig. 7-9. In the four transductant clones tested, three of the clones contain the plasmid pUWM5D:: $\Omega$ . The remaining clone appears to contain an integrated multimer of pUWM5D. This contains no sequences from the omega fragment. The three clones containing the omega fragment exhibit high levels of  $\beta$ -galactosidase activity during sporulation as compared to  $\beta$ -galactosidase activity during vegetative growth. A smaller rise is apparent in the fourth clone DK306[pUWM5D:: $\Omega$ ](4) (Table 7-6). It is conceivable that the clone lacking omega sequences has lost them as a result of an internal deletion event. This could have deleted some of the M. xanthus sequences required for developmentally regulated expression. Such a deletion, if small, would not be apparent on the Southern blot. However, the one assay of a single clone is hardly convincing evidence for the loss of sporulation dependent regulation. There is normally considerable variation in the degree of glycerol induction in strains carrying pUWM5. Even so, the absence of omega sequences in one clone is not easy to account for in any way other than the spontaneous deletion of omega. One of the four transductants (DK306[pUWM5D:: $\Omega$ ](1)) appeared to be a mixed population of plasmids with omega and plasmid without omega. Excluding the possibility of accidental contamination, all four transductant strains were derived from a single E. coli clone (pUWM5D:: $\Omega$ ). The omega sequences may well have been lost either during or

Fig. 7-8

Southern analysis of *M. xanthus* strains obtained by transduction with pUWM5D:: $\Omega$ .

## Track:

- |   |                              |
|---|------------------------------|
| 1 | OK306[pUWM5D:: $\Omega$ ](1) |
| 2 | OK306[pUWM5D:: $\Omega$ ](2) |
| 3 | OK306[pUWM5D:: $\Omega$ ](3) |
| 4 | OK306[pUWM5D:: $\Omega$ ](4) |
| 5 | HindIII digested lambda DNA  |

- a. Probe: pUWM5' +  $\lambda$ DNA
- b. Probe: pDAH122
- c. Probe: purified omega fragment

In hybridisation c. the nitrocellulose was not stripped of probe after hybridisation b. but the radioactivity had decayed over three months. Consequently, faint bands are still visible from hybridisation b..

The sizes are indicated for the marker fragments of lambda DNA digested with HindIII and EcoRI. The track of the EcoRI digest was in another part of the Southern blot not shown here.

The band sizes from this Southern blot are presented in Table 7-7.

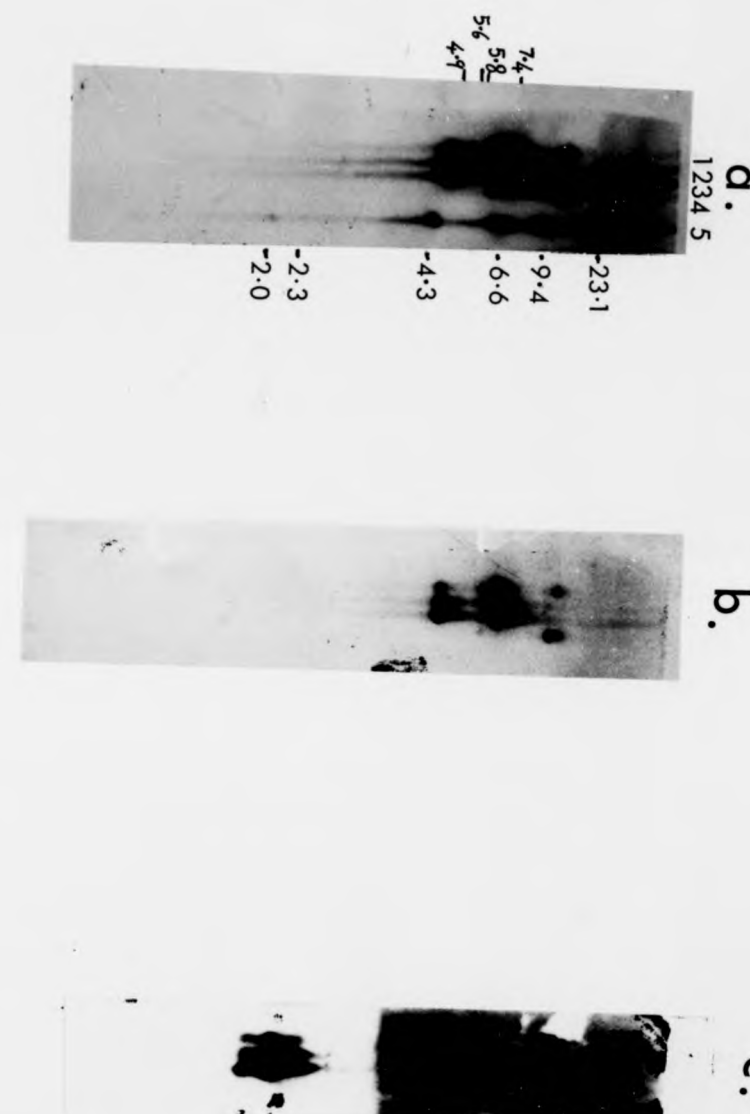


Fig. 7-8



Fig. 7-9

Interpretation of Southern analysis of strains transduced by PUWMSD:: $\Omega$

a. shows the omega insert within the gene fusion.

b. shows the gene fusion with no omega insert.

In both cases the plasmids have integrated as multimers (here shown as dimers).

The intact gene and gene fusion to *lacZ* are both shown as unshaded boxes.

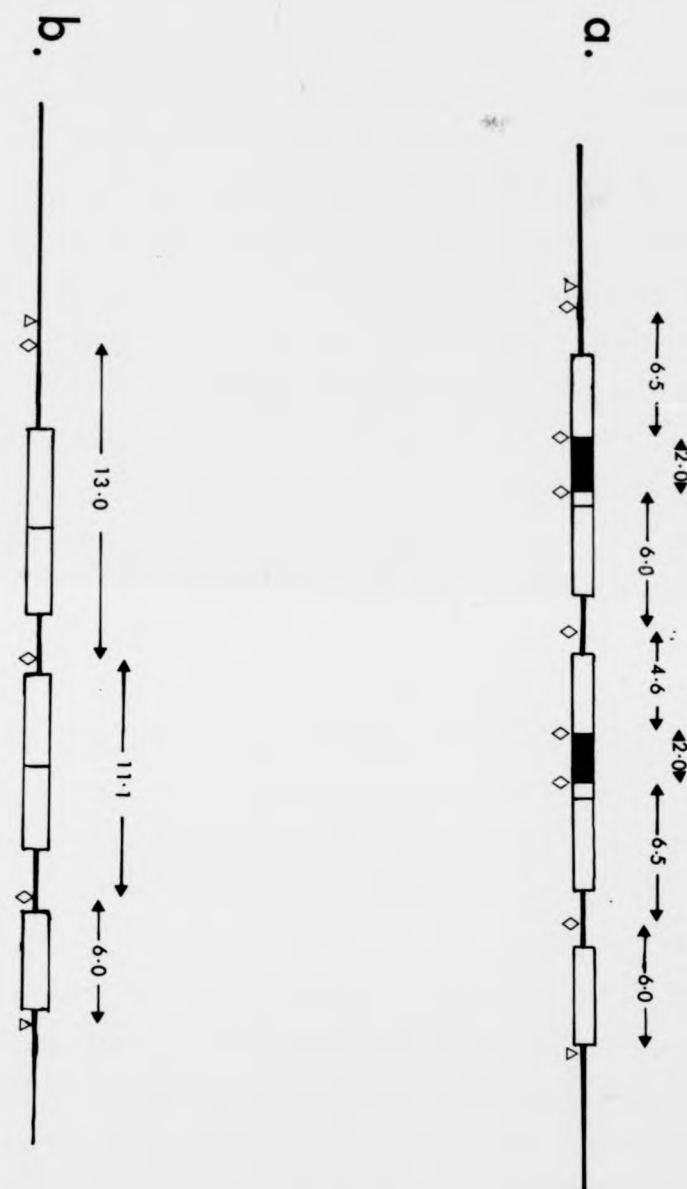
The omega fragment is shown darkly shaded.

Open triangle symbols represent *Bam*HI sites.

Open diamond symbols represent *Hind*III sites.

The arrows above each map indicate the fragments hybridising to plasmid PUWMS' and also to pDAH122. The exception is the 2.0kb fragment hybridising of the omega fragment. This hybridises instead to an omega specific probe.

Fig. 7-9





after transduction.

The fact that the inducibility of the gene fusion was unaffected by an omega insert just 700bp upstream of the fusion point suggests that the sequences required for glycerol inducibility lie within a 700bp region. In two of the transductant strains there was no sign on the Southern of any gene fusion without an omega insert. However, the integrated plasmid is multimeric and so it is just possible that a single copy sequence lacking an omega sequence might escape detection by Southern analysis. However, this is unlikely in view of the magnitude of response in the two transductant strains.

#### 7-6 Concluding remarks

The attempts to map gene promoters using site directed mutagenesis of the promoter probe plasmid were partially successful. One problem encountered was spontaneous loss of the insert used for mutagenesis. This was the case where no selection for the insert was possible in M. xanthus. The other problem was the tendency for a promoter probe plasmid to integrate as a multimer. This was correlated with the lack of P<sub>linc</sub> sequences in the plasmid and also with the size of the plasmid. For example, the plasmid pUWM5':::Tn5(-1300) contains P<sub>linc</sub> sequences, and always integrated as a monomer. By contrast, the plasmid pUWM5D::Ω, which lacks P<sub>linc</sub> sequences appeared to integrate as a high copy multimer. The pUWM4Dy6 plasmids integrated mostly as monomers, although in a minority of cases, dimers were formed. These plasmids contain no P<sub>linc</sub> sequences and yet still integrated as monomers. The size of

the plasmids (16kb) is well below the packaging limit of P1 (45kb). The existence only of monomers could be due to the internal resolvase encoded by gamma-delta. This could prevent multimers from forming in the first place. This would also explain why most transductants contained no gamma-delta. If a small population of plasmids contained no gamma-delta, these alone would have been able to form multimers. The only stable multimers would be those containing either no gamma-delta or one copy only. Such plasmids would appear preferentially among the transductants. However, this is not a wholly sufficient explanation. Even where a transductant lacked any gamma-delta sequences, the plasmid was present in one or, at most, two copies. This contrasts with the large multimers of plasmids observed when pUWMSD:: $\Omega$  became integrated into the M. xanthus chromosome. One possible reason for the lack of large multimers after transduction with pUWMSD:: $\Omega$  would be if multiple copies of the gene were harmful to the cell. Any intact gene in the isgA1 region is likely to be overexpressed under these circumstances. Overexpression could, conceivably, also occur through insertion of gamma-delta with its outwardly directed promoters. This would explain why one clone containing an insert of gamma-delta could be transduced into M. xanthus while three others could not be transduced without loss of the gamma-delta sequences. In any circumstances, the existence of outward reading promoters in gamma-delta makes it unsuitable for mutagenesis of gene fusions.

Attempts to locate promoter sequences by mutagenesis of the gene fusion isgB2>lacZ were much more successful. A InS insert 1.3kb upstream of the fusion point had no effect upon the expression of the gene during glycerol inducible

sporulation. A similar experiment where the omega fragment was used for insertional mutagenesis, was harder to interpret. Here, the plasmid integrated as a multimer. This made the Southern analysis of transductants harder to interpret. Furthermore, no selection was attempted for the omega fragment once it had been transduced into M. xanthus. In spite of these limitations, there was a strong indication that the developmental regulation of the promoter was unaffected by an insert only 0.7kb upstream of the fusion point. Both experiments with isg82>lacZ indicate the limits of a region containing the promoter together with any other sequences which may be involved in developmental regulation. It is extremely unlikely that sequences further upstream could direct transcription into isg82>lacZ. Both Tn5 and the omega fragment are of considerable length and both contain transcription terminators. The results do not rule out the possibility that regulatory sequences might be able to act over a distance, for example by altering the degree of supercoiling. In this way, upstream or downstream sequences could act upon gene expression even after insertional mutagenesis.

## CHAPTER 8

Overall discussion: The  
relevance of results obtained  
to the understanding of  
microbial development;  
possible future experiments.

8-1 The promoter-probe library of M. xanthus  
chromosomal DNA

Gene libraries were constructed in promoter-probe plasmids which could be cloned in E. coli. The gene libraries were then successfully transduced into Myxococcus xanthus. The level of variation between transductant strains in the expression of  $\beta$ -galactosidase was considerable. Over 6000 independent clones were obtained in E. coli (Table 3-3). From these, over 4000 transductant clones were obtained in M. xanthus. The preparation of gene fusions in a two-step process such as this, might be expected to be inefficient compared to a one-step process such as Tn5 mutagenesis. In a large number of transductants obtained from bulk transduction with a plasmid library packaged with P1, some plasmids are likely to be represented more frequently than others. This could be a random statistical process. It is also possible that the bias could be increased if certain plasmid inserts affected the growth rate or viability of the host E. coli or host M. xanthus. For this reason, for every 1000 recombinant clones obtained in E. coli, a much larger number of M. xanthus transductants will need to be tested in order to ensure that most of the gene fusions are screened. No attempt was made to find how representative the M. xanthus transductants were of either the library initially obtained in E. coli or how representative the gene fusions were of the M. xanthus chromosome. Possible future work could make use of colony hybridisation using known M13 clones of M. xanthus DNA as

probes. The frequency of occurrence of the hybridising sequence could be compared with that predicted from the size of the genome.

The advantages of using the plasmid based promoter-probe may outweigh the possible shortcomings described in the previous paragraph. It is possible using this method, to prepare gene fusions to essential genes. Such gene fusions would be expected to be largely absent from a library of Tn5 generated gene fusions unless the transposon was situated between the translation and transcription termination sites. Indeed, evidence is presented in Chapter 6 that the lsgA1 gene is an essential gene. Although screening for gene fusions of interest may take slightly longer due to the inevitability of duplicate strains appearing in the transductants, there is a compensating advantage in the ease at which chromosomal gene fusions can be cloned.

#### 8-2 The screening procedures employed

Transductants were tested for  $\beta$ -galactosidase expression under both vegetative and sporulation conditions. The fluorogenic indicator 4-MUG was used as an alternative to X-gal. This provides an indication of  $\beta$ -galactosidase activity at a particular instant as opposed to the cumulative indication obtained as a blue colour on X-gal plates. Compared with the results of Kroos L. et al (1986) the number of strains showing large increases in expression was, perhaps, disappointingly

small. In those experiments, 2374 transductants were tested and 36 transductant strains were found which showed a greater than threefold increase in  $\beta$ -galactosidase expression during sporulation. By contrast, in our experiments only two strains: UWM4 and UWM5, were found to exhibit large increases in expression. This was in spite of the fact that the number of gene fusions screened was comparable to that in the experiments of Kroos L. et al (1986). One possible reason might be that the number of gene fusions was smaller than it appeared to be, owing to overrepresentation of certain clones among the transductants. This potential problem was described in the previous section. However, another likely possibility was that the screening procedure was more rigorous. Expression in cells grown on minimal medium was compared with expression in cells grown on TM medium. By contrast, Kroos et al. compared the expression of cells grown on Casitone medium with cells grown on starvation (TMP) medium. The latter method may have induced changes in gene expression which were strictly nutritional and of no direct relevance to sporulation. However, it should be mentioned that <sup>in</sup> their experiments, the strains which showed the greatest increase in activity were those where activity was confined to spores. This would suggest that these were fusions to genes involved in sporulation.

### B-3 Expression of gene fusions in different backgrounds

The ease at which the gene fusions could be cloned as a result of plasmid looping out of the chromosome proved very useful. The plasmid obtained carried the gene fusion to lacZ, together with P<sub>1</sub>inc sequences. This enabled the gene fusion to be repackaged in P1 and transduced into any chosen strain. The results were described in Chapter 4. An unexpected but highly significant result was obtained when gene fusion isgB2>lacZ (in plasmid PUWMS) was transduced into a nonmotile mgI<sup>-</sup> background. Expression of  $\beta$ -galactosidase in this strain increased enormously during developmental sporulation. By contrast, a strain with wild type motility into which the gene fusion had been transduced, showed hardly any increase. The results are best explained by assuming that M. xanthus has two different types of spore: one which forms outside of fruiting bodies, and another which forms within fruiting bodies. Another interpretation is that gene isgB2 is part of an auxiliary pathway in sporulation. It is not required in a fully motile background but is required in a nonmotile background. In the absence of fruiting bodies, the cells' microenvironment may be quite different than that within fruiting bodies.

Whichever of the two interpretations is correct, they would be evidence for alternative developmental pathways in M. xanthus. This would be a similar case to that in Bacillus subtilis. Here, there is a clearly defined



mother cell and prespore. Experiments with gene fusions have revealed that some genes activated during development show activity in the prespore only and others in the mother cell only (Losick R. et al. 1989). The difference with M. xanthus is that here, there are two spore types rather than one spore and one mother cell. Possible future work might include a detailed structural analysis and electrophoretic analysis of spores formed outside of fruiting bodies in order to compare them with spores formed within fruiting bodies. The gene fusion isgB2>lacZ is also switched on in glycerol induced spores. It might be expected, therefore, that spores formed outside of fruiting bodies might share some of the unusual characteristics of glycerol induced spores such as the thin spore coat or the detectable levels of respiration in the dormant spore.

Spores forming in the absence of the differentiation stages with which they are normally associated is a phenomenon encountered in other prokaryotes as well. In the case of the myxobacterium Stigmatella aurantiaca spores form in the undifferentiated cell swarm if fruiting bodies are prevented from forming (Stephens K., White D. 1980). In Streptomyces coelicolor spores can be formed in the substrate mycelium rather than in the usual position borne upon aerial mycelium. This occurs in strains overexpressing a sporulation specific sigma factor (Mendez C. and Chater K.F. 1987). In one strain of Streptomyces griseus, spores can be obtained in liquid cultures in the absence of a solid surface (Ochi K. 1987).

The specificity of gene lsgB2 for spores outside of fruiting bodies is not, however, complete. Strain DK101 was used as a background and found to support high levels of expression during sporulation, although not as high as those encountered in the mgI<sup>-</sup> strain DK306. It should be noted that DK101 contains a motility mutation sglA1 which decreases social motility without abolishing it. This is a significant finding as this is the strain in which many sporulation mutants were first isolated (Hagen *et al.* 1978). Such spores, therefore, may not be typical of spores in wild type (DK1622) M. xanthus. Possible future experiments might include examination of expression of the gene fusion in circumstances where sporulation was hindered and perhaps delayed, without being blocked altogether. Sporulation could be delayed either by genetic background, for example a frizzy mutation, or by external physical or chemical means such as the addition of Congo Red or EGTA (Ethylene glycol tetracetic acid).

The fusion lsgB2>lacZ was tested in sporulation blocked mutants. Some increase was observed here, although the increase was much less than in a DK306 background. These increases were quite small, about twofold. Consequently, the experiments would need to be repeated in order to confirm the significance of these results. Nevertheless, in the Dsg strain DK3621 there is a quite convincing increase in expression when under starvation conditions (Fig. 4-1-4). It is possible that there is a partial dependence upon expression of the dsg gene.

The gene fusion lsgA1>lacZ shows increased expression

during starvation conditions regardless of genetic background. The gene fusion is also strongly expressed in vegetative cells growing in minimal medium. It is possible that this gene has a biosynthetic function for a nutrient present in Casitone medium but not in minimal medium. Further experiments might be able to identify this nutrient. The expression of the gene is probably also associated with sporulation. This is revealed by the extremely high levels of expression in spores induced not by starvation but by addition of glycerol. There may, therefore, be an increased demand for the nutrient during sporulation.

The gene fusion from strain UWM2 was also tested in a variety of backgrounds. It was more difficult to obtain convincing results with this gene fusion because the increase in expression during sporulation was relatively low (two to threefold). However, it appeared that neither of the two *Asg* strains (DK5057 and DK4727) could correctly regulate expression of the gene fusion. The latter strain showed remarkably low levels of vegetative expression. It would be worth repeating the experiment to confirm that finding. As for the one other sporulation mutant tested, a *Dsg* strain (DK3621) did show a starvation induced increase in expression of the gene.

8-4 Use of site directed mutagenesis in determining the functional regions of genes and the position of gene promoters

Mutagenesis experiments upon the lsgA1 and lsgB2 regions were carried out using transposon Tn5. No mutants could be obtained targeted to the lsgA1 region. By contrast, mutants were readily obtained from the lsgB2 region. This may be because lsgA1 is an essential gene. There are other possible explanations. For example, the plasmid pUWM4' used as a mutagenesis target lacked P<sub>lnc</sub> sequences. Consequently, only concatameric plasmid would have been packaged. This may somehow have prevented gene replacement events. Subsequent experiments could involve insertion of the P<sub>lnc</sub> region into the plasmid. Mutant strains containing inserts targeted to the lsgB2 region had no apparent mutant phenotype. This could be because the coding region of the gene was missed. Another possibility could be that there was not an absolute requirement for the gene in sporulation. This interpretation would be consistent with other work with gene fusions in the literature. In M. xanthus (Kroos L. et al. 1986), only a small proportion of developmentally regulated Tn5::lac insertions showed any obvious mutant phenotype. In Bacillus subtilis likewise, transposon inserts within developmentally regulated transcription units rarely have any effect upon sporulation (Losick R. et al. 1989). The implication is that any one spore component has a subtle and largely dispensable role in spore formation. A more rigorous and quantitative assay

of spore numbers may, in the future, reveal a mutant phenotype associated with the isg82 gene.

Mutagenesis was also used in attempts to locate the position of the promoters of the isgA1 and isg82 genes. Insertional mutations were obtained of gene fusions isgA1>lacZ and isg82>lacZ. In many of these experiments the plasmid size was reduced and the P1inc sequences removed. The resulting plasmid inserted into the M. xanthus chromosome as a concatamer. Future experiments could use transformation by electroporation rather than P1 transduction. It would be possible then to introduce small plasmids in monomeric form without the need for P1inc sequences. This would eliminate the complications produced by having multiple copies of a gene. In spite of these shortcomings, there was substantial evidence that the promoter of isg82 was situated within 700bp of the fusion point. It would be desirable to obtain transposon inserts closer still to the fusion point. If the promoter was found to be less than 200bp from the fusion point then the mutations already obtained targeted to the chromosomal copy of the gene would not be inserts within the gene. If the promoter were to be found between 200 and 700bp from the fusion point then the chromosomal insert at the point corresponding to 200bp upstream of the fusion point should inactivate the gene.

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